ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

# World Journal of *Gastroenterology*

*World J Gastroenterol* 2018 April 21; 24(15): 1583-1678

Published by Baishideng Publishing Group Inc

World Journal of Gastroenterology

Contents

Weekly Volume 24 Number 15 April 21, 2018

**Contents**

*World Journal of Gastroenterology*
**Volume 24 Number 15 April 21, 2018**

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NAME OF JOURNAL
*World Journal of Gastroenterology*

ISSN
ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

LAUNCH DATE
October 1, 1995

FREQUENCY
Weekly



EDITORIAL OFFICE
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*World Journal of Gastroenterology*
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7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-2238242
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7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
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Fax: +1-925-2238243
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PUBLICATION DATE
April 21, 2018



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World Journal of Gastroenterology

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*World J Gastroenterol* 2018 April 21; 24(15): 1583-1590

DOI: 10.3748/wjg.v24.i15.1583

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

REVIEW

# Ultrasound findings in autoimmune hepatitis

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Author contributions: All authors equally contributed to this paper with conception and design of the study, literature review and analysis, drafting and critical revision and editing, and final approval of the final version.

Conflict-of-interest statement: No potential conflicts of interest. No financial support.



Manuscript source: Unsolicited manuscript

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Received: February 1, 2018
Peer-review started: February 2, 2018
First decision: February 24, 2018
Revised: March 20, 2018
Accepted: March 30, 2018
Article in press: March 30, 2018
Published online: April 21, 2018

## Abstract

Ultrasound findings in autoimmune hepatitis (AIH) have not been reported systematically so far. The use of reliable and accurate noninvasive methods for determining fibrosis stage is important in evaluation of treatment efficacy and fibrosis regression in AIH. Imaging plays an important role in detection of complications and ruling out other possible causes of chronic liver diseases. Ultrasound elastography cut-off values in AIH patients are not the same as those in patients with chronic viral hepatitis or non-alcoholic fatty liver disease. AIH is characterized by wide fluctuations in inflammatory activity. Here we report on current knowledge of ultrasound findings in AIH.

**Key words:** Autoimmune hepatitis; Fibrosis stage; Ultrasound; Elastography; Chronic liver diseases



**Core tip:** Accurate noninvasive imaging to determine fibrosis stages is of importance in the evaluation of treatment efficacy and fibrosis regression in autoimmune hepatitis (AIH). The cut-off values in AIH patients are not the same as those in patients with chronic viral hepatitis or non-alcoholic fatty liver disease.

Dong Y, Potthoff A, Klinger C, Barreiros AP, Pietrawski D, Dietrich CF. Ultrasound findings in autoimmune hepatitis. *World*

*J Gastroenterol* 2018; 24(15): 1583-1590 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i15/1583.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i15.1583

## INTRODUCTION

Autoimmune hepatitis (AIH) is a chronic immune mediated liver disease of unknown etiology[1,2]. About one-third of the patients already have developed advanced fibrosis and liver cirrhosis at the time of diagnosis. AIH mainly affects women and is usually characterized by chronic inflammation of the liver, hypergammaglobulinemia with increased immunoglobulin G (IgG) levels and circulating autoantibodies associated with human leukocyte antigens DR3 or DR4, typical liver histology with interface hepatitis[3], and a favorable response to immunosuppressive treatment[1,2,4]. Once other liver diseases such as viral hepatitis have been excluded, the diagnosis of AIH can be made by serological and histological findings. AIH can range from a mild or severe course to fulminant hepatic failure. Despite corticosteroid therapy, hepatic fibrosis develops in 25% of patients with AIH[5]. To find reliable and accurate noninvasive imaging methods for determining fibrosis stages is of importance in the evaluation of treatment efficacy and fibrosis regression in AIH[6]. Here we report on ultrasound findings in AIH.

## CLASSIFICATION OF AIH

AIH has a global distribution. It is considered as a rare disease affecting all ages and ethnic groups with a female predominance (F:M ratio 3.6:1). The incidence of AIH is around 1 per 100000 persons per year[7]. In 1992, the International AIH Group (IAIHG) reported diagnostic criteria[8], which were remarkably simplified in 2008[9]. AIH is classified into two major types: AIH type 1 (AIH-1) and AIH type 2 (AIH-2). Antinuclear antibodies (ANA) and/or smooth muscle autoantibodies (SMA) could be detected in AIH-1. Also perinuclear anti-neutrophil cytoplasmic antibodies (p-ANCA) could be detected in 60%-90% of AIH-1 patients[4,5,9,10]. AIH-2 is characterized by the detection of anti-liver/kidney microsomal antibody type 1 or anti-LKM type 3[4,10] and/or antibodies against liver cytosol type 1 antigen[1,4]. AIH-1 accounts for about 75%-80% of all patients, however, AIH-2 is more frequently seen in children and young patients, which might present with acute onset and severe histological changes at time of diagnosis. Poor treatment prognosis, recurrence after treatment and need for lifelong treatment are more common in AIH-2[4,11]. AIH-1 patients might also show antibodies against soluble liver/liver-pancreas-antigen SLA/LP-[12,13].

## PATHOGENESIS

According to the dominant pathogenetic hypothesis, AIH develops in genetically susceptible individuals by several triggers. The liver is attacked through mechanisms of "molecular mimicry", and is promoted by down regulation of regulatory T-cells[3, 4].

AIH may develop after the use of some drugs and biological agents or after viral infections and other events, including *de novo* after orthotopic liver transplantation[14-16]. AIH may first develop during pregnancy and after delivery.

## CLINICAL MANIFESTATIONS

Clinically AIH is characterized by fluctuation of disease activity. Its clinical symptoms range from no obvious manifestations to severe and acute hepatitis [4,17]. Clinical manifestations range from merely elevated transaminases to liver cirrhosis and/or fulminant liver failure requiring liver transplantation[18]. Acute AIH presents in approximately 25% patients with similar symptoms as patients suffering from acute toxic or viral hepatitis[19]. At time of diagnosis, about one third of patients have established cirrhosis[3]. A specific and common clinical characteristic of AIH is its association with other autoimmune diseases including first degree relatives[20]. Concurrent extrahepatic autoimmune conditions mostly affect the thyroid gland (10%-23%)[13]. Clinical presentation of AIH might be similar to primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC). These diseases may coexist leading to overlap or variant syndromes[21,22].

## DIAGNOSIS

Due to the absence of specific diagnostic features and diversity of clinical manifestations, serological and histological features, AIH diagnosis may be a challenge[3]. According to the International Autoimmune Hepatitis Group (IAIHG), the clinical diagnosis of AIH is based on biochemical, immunological, and histological features. Viral hepatitis should be excluded[9]. The simplified diagnostic criteria of IAIHG for AIH is based mainly on four parameters, including autoantibodies detection, serum IgG levels, absence of viral hepatitis markers and liver histology[9]. Histological changes including interface hepatitis, and hepatic rosette formation and emperipolesis[9]. Autoantibodies detectionis regarded as the hallmark for a timely diagnosis although not pathognomonic[3].

## LABORATORY ASSESSMENTS AND LIVER BIOPSY

Liver biochemistry is not characteristic in most of AIH patients, with elevated bilirubin and transaminases. In most patients, polyclonal hypergammaglobulinemia with particular elevated level of serum IgG is observed. However, it should be mentioned that 15%-25% of patients (especially children, elderly and acute cases) have normal IgG levels. Therefore, AIH diagnosis

should not be excluded depending on a normal IgG testing[3]. The standard laboratory assessments include elevated LFTs, hypergammaglobulinemia, and the detection of autoantibodies (ANA, anti-SMA, and anti-LKM).

Liver biopsy is strongly recommended to confirm AIH[13], first to make the diagnosis and second to determine the stage of disease. The diagnostic histological features of AIH include moderate to severe interface hepatitis without biliary lesions or well-defined granulomas. However, it must be noted that pathognomonic histologically characteristics for AIH are missing. Regular assessment of hepatic fibrosis is important in patients with AIH because progressive fibrosis ultimately leads to cirrhosis and liver failure[23]. It has been recommended that clinical decisions about duration of treatment or immunosuppressive therapy should be based on clinical remission and histological features[2].

## NON-INVASIVE MARKERS OF LIVER FIBROSIS

Laboratory methods can differentiate liver cirrhosis from non-cirrhosis, but their accuracy in distinguishing changes of AIH in histological stages is uncertain. Biochemical markers can reflect the therapeutic response during treatment, but they cannot reflect the severity of liver fibrosis[5]. Many non-invasive markers for assessing liver fibrosis and cirrhosis have been applied in clinical practice[24,25]. However, their ability to detect early stages of liver fibrosis and cirrhosis in AIH patients is still uncertain[26]. All calculated non-invasive markers are not specific. However, it has been consider feasible to predict the degree of liver fibrosis in patients with AIH using laboratory parameters. Platelet count as well as AAR could be used to predict the presence of advanced fibrosis[27,28].

## DIFFERENTIAL DIAGNOSIS

Differential diagnosis of AIH includes chronic viral hepatitis (B and C), primary sclerosing cholangitis, alpha-1 antitrypsin deficiency, primary biliary cirrhosis, hemochromatosis, Wilson's disease and drug induced hepatitis (*e.g.*, minocycline, nitrofurantoin, isoniazid, methyldopa). However, to differentiate AIH from drug-induced liver injury (DILI) might be a challenge in cholestatic and severe clinical presentations, in particular when circulating liver autoantibodies are detectable in serum[2] Elevated IgG serum-levels and the histological presence of plasma cells can be found as well in a significant proportion of DILI patients[29].

### *Treatment*

In order to prevent progressive liver fibrosis/cirrhosis, treatment aims on complete biochemical (defined by normalization of aminotransferases and IgG level) and histological remission[13,30]. Most patients respond well to immunosuppressive therapy, which usually results in an excellent prognosis[1,31,32]. Steroids are used as initial therapy leading to a treatment response in 80% of patients with AIH[33-35]. In adults, Azathioprine is effective as maintenance therapy[5,10,30]. Treatment should be continued until normalization of laboratory tests and liver histology[36]. Incomplete response and treatment failure occur in 14%[37] and 7% of patients[38], respectively[36,39,40]. Treatment failure is characterized by a missing decrease of aminotransferase levels and, in some patients, rapid progression to cirrhosis. Consequently, alternative therapeutic regimens have to be considered[5,41,42]. In cases of treatment failure, overlap with other etiologies should be considered. In those patients with liver failure, liver transplantation might be indicated and carries a 10-year survival rate exceeding 70%[1]. Future anti-fibrotic therapies and monitoring fibrosis progression are essential in patients with in AIH.

## ULTRASOUND IMAGING

### *B-mode ultrasound and contrast-enhanced ultrasound*

No characteristic conventional ultrasound imaging features of AIH have been described. For initial diagnosis of AIH, ultrasound, computed tomography (CT), and magnetic resonance imaging (MRI) are valuable methods to detect liver cirrhosis and its complications. Imaging of AIH play a role in the detection of complications[21]. Enlarged perihepatic lymph nodes are a typical ultrasound feature, similar to virus hepatitis C[43-46] (Figure 1), PBC[47,48], PSC[49], sarcoidosis[50,51] and other inflammatory liver diseases[50] in adults and children[46,50] (Figures 1 and 2). These enlarged inflammatory perihepatic lymph nodes show typical contrast behavior and elastographic architecture[52-59] (Figure 3).

About 1%-9% of AIH patients with liver cirrhosis develop HCC. Therefore, ultrasound follow-up examinations are recommended every six month[60]. Characteristically, involvement of the biliary tract is absent or minimal in AIH. Magnetic resonance cholangiography (MRC) is recommended in all children and adult patients with elevated markers of cholestasis in order to detect concurrent overlap syndromes, particularly PSC.

### *Ultrasound elastography*

Non-invasive liver ultrasound elastography methods are useful for detection and staging of liver fibrosis initially as well as during clinical follow-up. Transient elastography (TE) has been introduced first to assess liver stiffness in patients with chronic liver diseases[61,62]. Other newer ultrasound-elastography methods include point shear-wave elastography (pSWE) and two-dimensional shear-wave elastography (2D-SWE)[63]. These tools are integrated in standard ultrasound devices.

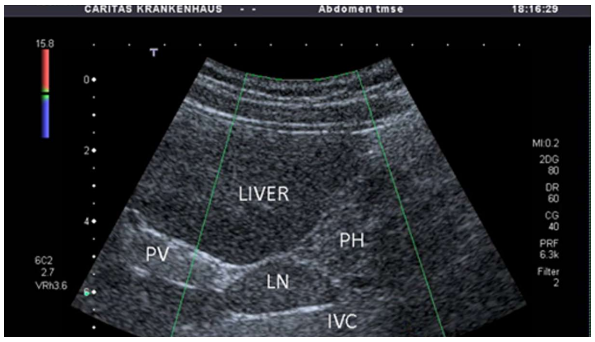


**Figure 1 Enlarged perihepatic lymph nodes dorsal in the hepatoduodenal ligament between the portal vein and inferior vena cava is a typical sonographic sign of autoimmune hepatitis.** PV: Portal vein; PH: Pancreatic head; ICV: Inferior vena cava.

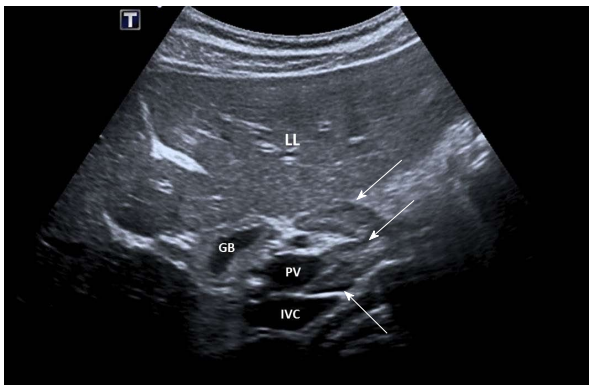


**Figure 2 Enlarged perihepatic lymph nodes ventral and dorsal in the hepatoduodenal ligament between the portal vein and inferior vena cava (white arrows).** LL: Liver; GB: Galbladder; PV: Portal vein; ICV: Inferior vena cava.

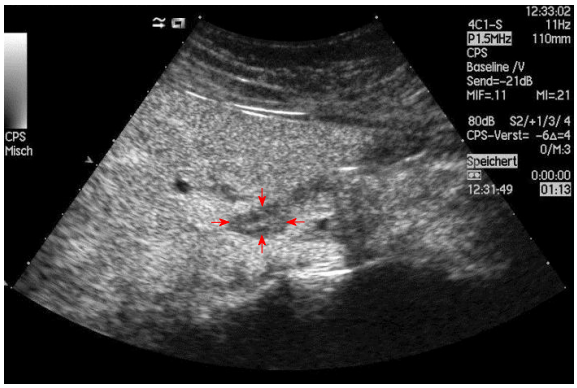

**Figure 3 Enlarged perihepatic lymph nodes dorsal in the hepatoduodenal ligament is a typical sonographic sign of autoimmune hepatitis.** Contrast enhanced ultrasound shows normal lymph node architecture (in between arrows).

Ultrasound elastography methods are proved to be accurate and reliable in the diagnosis of advanced fibrosis and cirrhosis, however, the diagnostic performances may be compromised by inflammation, congestion, biliary obstruction and obesity[63]. Magnetic resonance elastography (MRE) has excellent performance parameters for all histological stages in diverse liver diseases[64], which represents to be a reliable alternative to SWE. MRE is less influenced by body habitus and inflammatory activity in the evaluation of fibrosis in AIH[65-67] but its availability is limited and the investigation is more expensive.

### *Transient elastography*

The cut-off values in AIH patients are not the same as those in patients with chronic viral hepatitis. A recent study which enrolled 108 AIH patients who underwent liver biopsies, AUROC value of liver stiffness measurement (LSM) was 0.885 for stage F2 ($n$ = 24), 0.897 for stage F3 ($n$ = 30), and 0.878 for stage F4 ($n$ = 24). The optimal LSM cut-off value was 6.27 kPa for stage F2, 8.18 kPa for F3, and 12.67 kPa for F4[68]. LSM was superior to other non-invasive markers in differentiating the stages of fibrosis in AIH patients[68]. Liver stiffness measured by TE correlated significantly with the stage of liver fibrosis in a study which compared accuracy of TE and liver biopsy in AIH patients. TE correlated better than non-invasive laboratory markers[69,70]. This study demonstrated similar cut-off values, with LSM cut-off values of 6.45 kPa for F2, 8.75 kPa for F3, and 12.5 kPa for F4[70].

A previous study[70] evaluated the accuracy of LSM, APRI, and FIB-4 in 100 AIH patients. TE outperformed the other non-invasive markers. LSM was proven to be closely associated with fibrosis stages ($r$ = 0.752, $P$ < 0.01). Patients with more advanced fibrosis stages are associated with higher LSM values. Of importance, serum ALT levels had minor effect on LSM values and hepatic inflammatory activity had no significant effect on LSM determination.

TE proved also to be more accurate than APRI score in study published by Halasz *et al*[71] including 22 cases of AIH.

Wang *et al*[69] conducted a retrospective study with 36 histologically confirmed AIH patients (19 treated and 17 untreated). They reported that TE was accurate for distinguishing hepatic fibrosis in AIH between stages F0-F2 and F3-F4.

While comparing to other etiologies, the higher LSM values for different Ishak stages in AIH patients are in line with the results in the literature[69,72-74]. In a pediatric study of Behairy *et al*[6], a total of 90 children (HCV $n$ = 50, AIH $n$ = 20, Wilson´s disease $n$ = 20) were included and underwent LSM using TE. AIH patients had both higher values of LSM and (necro) inflammation scores compared to patients with HCV and Wilson´s disease. Inflammatory activity accompanying with increased serum aminotransferase levels, can increase liver stiffness may be misinterpreted as fibrosis[75,76]. Therefore, the higher grade of (necro-) inflammatory activity in AIH patients compared to other etiologies could be a possible explanation[2,3].

Long-term treatment with mono corticosteroids or in combination with azathioprine is proposed when the AIH diagnosis is established. The effect of treatment on the diagnostic performance of LSM has been studied

as well. Hartl *et al*[77] reported that performance of TE in the detection of cirrhosis is better for AIH patients who received longer treatment compared to treatment-naïve patients and patients with shorter duration of treatment. Using the cut-off of 16 kPa, the diagnostic accuracy for cirrhosis was excellent in patients ($n$ = 36) under immunosuppressive treatment for 6 months or longer[77]. A non-invasive inflammatory score has been proposed to discriminate patients with and without significant hepatic inflammation[78]. Those scores are easy to calculate, however, they would be only suitable to patients without co-morbidities and not for patients with low inflammatory activity[79]. Weight gain is a common consequence of corticosteroid treatment[80,81].

### *Acoustic radiation force impulse imaging*

Acoustic radiation force impulse imaging (ARFI) can help to distinguish liver fibrosis patients with autoimmune liver diseases from healthy subjects[82,83]. In AIH patients after at least 2 years of biochemical remission, ARFI allowed to differentiate significant (F ≥ 2) from non-significant liver fibrosis (F < 2) (2.28 ± 0.68 m/s *vs* 1.20 ± 0.24 m/s, $P$ = 0.002)[23]. Although large studies on ARFI elastography in AIH patients are still lacking preliminary data indicate that ARFI is a promising non-invasive method for detection and staging of fibrosis also in AIH patients.

### *2D-SWE*

SuperSonic shear wave imaging (SuperSonic Imagine, Aix-en-Provence, France) had higher values in liver fibrosis with AIH of stages S2-S4[84] similar to the results with ARFI[82]. The shear moduli were 9.41 ± 2.5 kPa in S0 stage; 10.42 ± 5.1kPa in S1 stage; 13.25 ± 5.6 kPa in S2 stage; 19.03 ± 7.8 kPa in S3 stage and 24.99 ± 9.5 kPa in S4 stage[84].

### *Real-time elastography*

In an animal based study, Hao *et al*[85] investigated the inflammation effect on fibrosis staging by measuring quantitative elasticity parameters in AIH rats (HiVision Preirus, Hitachi Medical Systems Co, Ltd, Tokyo, Japan). The grade of inflammation will influence the accuracy of Real-time elastography (RTE) measurements. The liver fibrosis index had the highest correlation with inflammation grading ($r$ = 0.766; $P$< 0.05).

## LIMITATIONS

Currently, ultrasound findings in AIH have been limited use so far. No characteristic ultrasound imaging features of AIH have been described in the literature. There is a need for studies to determine the better use of ultrasound in AIH patients.

## CONCLUSION

In conclusion, AIH is characterized by wide fluctuations in inflammatory activity, Thus, stage of fibrosis can be overestimated by transient elastography[86] due to concomitant hepatic necroinflammatory activity. It can be also concluded that LSM using TE reflects the stages of liver fibrosis and correlates better than non-invasive laboratory markers in patients with treated AIH[77,87,88]. Other non-invasive ultrasound based techniques as ARFI, 2D-SWE or Real Time Elastography are not well investigated in the population of AIH patients yet. Further studies are needed. However, current non-invasive markers/methods for the evaluation of liver fibrosis in AIH could not replace liver biopsy, especially in differentiating mild from severe stages of fibrosis[87].

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**P- Reviewer**: Gatselis NK, Tai DI, Trifan A **S- Editor**: Ma YJ
**L- Editor**: A **E- Editor**: Huang Y

World Journal of Gastroenterology

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*World J Gastroenterol* 2018 April 21; 24(15): 1591-1600

DOI: 10.3748/wjg.v24.i15.1591

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

REVIEW

# Nutrition status and *Helicobacter pylori* infection in patients receiving hemodialysis

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Author contributions: Sugimoto M, Yasuda H and Andoh A wrote the paper.

Conflict-of-interest statement: No potential conflicts of interest.



Manuscript source: Invited manuscript

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Received: February 23, 2018
Peer-review started: February 23, 2018
First decision: March 15, 2018
Revised: March 18, 2018
Accepted: March 25, 2018
Article in press: March 25, 2018

Published online: April 21, 2018

## Abstract

Chronic kidney disease (CKD) patients receiving hemodialysis (HD) often develop gastrointestinal abnormalities over their long treatment period. In general, prognosis in such patients is poor due to the development of protein-energy wasting (PEW). Therefore, it is important to clarify the etiology of PEW and to establish better strategies to deal with this condition. Chronic *Helicobacter pylori* (*H. pylori*) infection in the gastric mucosa has a close association with not only the development of peptic ulcer disease and gastric cancer, but is also associated with abnormal plasma and gastric mucosal ghrelin levels that are seen in malnutrition. It is unclear whether *H. pylori* infection of the gastric mucosa is directly associated with prognosis in HD patients by affecting ghrelin levels. Recent studies show that the prevalence of *H. pylori* infection in HD patients is significantly lower than in subjects with normal renal function. In the natural history of *H. pylori* infection in HD patients, the prevalence of infection decreases as the length of time on HD increases. The severity of gastric mucosal atrophy has been suggested as the major determinant of ghrelin levels in these patients, and eradication therapy of *H. pylori* improves nutritional status by increasing serum cholinesterase and cholesterol levels, especially in patients with mild-to-moderate gastric mucosal atrophy. Prompt *H. pylori* eradication to inhibit the progress of gastric atrophy may be required to prevent this decrease in ghrelin levels and subsequent PEW and improve the prognosis of HD patients by improving their nutritional status.

**Key words:** *Helicobacter pylori*; Hemodialysis; Ghrelin; Gastric mucosa; Anti-bacterial agents

**Core tip:** Hemodialysis (HD) patients have a poor prognosis related in part to protein-energy wasting (PEW), associated with low levels of ghrelin. The severity of gastric mucosal atrophy has been suggested as the major determinant of ghrelin levels. Eradication of *Helicobacter pylori* (*H. pylori*) improves nutritional status, with serum cholinesterase and cholesterol levels stimulated by rising ghrelin levels and appetite, especially in *H. pylori* infection-positive patients with severe gastric mucosal atrophy. Although infection rates of *H. pylori* have been decreasing in HD patients, it would be preferable to eradicate *H. pylori* promptly before progression of gastric atrophy for prevention of gastric cancer and PEW.

Sugimoto M, Yasuda H, Andoh A. Nutrition status and *Helicobacter pylori* infection in patients receiving hemodialysis. *World J Gastroenterol* 2018; 24(15): 1591-1600 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i15/1591.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i15.1591

## INTRODUCTION

With ongoing progress in medical and dialysis machine techniques, the number of chronic renal failure patients receiving hemodialysis (HD) is increasing at a rate of 7% per year. At least 2.9 million Asians require dialysis, including Japanese, who live in an aging society and experience changes in their living environment[1,2]. HD patients often experience gastrointestinal symptoms (*e.g.*, nausea, abdominal pain, and constipation) caused by elevated urea levels, decreased gastrointestinal motility, amyloid protein deposition, and sensory disturbances, and are at increased risk of developing gastrointestinal diseases (*e.g.*, peptic ulcer disease, gastric antral vascular ectasia, gastroesophageal reflux disease, and gastric cancer)[3-7]. The risk of gastric mucosal damage is elevated in this population, in association with high ammonia levels[8], systemic and/or local chronic circulatory failure[9,10], and hypergastrinemia[11]. Gastroduodenal diseases such as peptic ulcer and gastric cancer have been linked to chronic *Helicobacter pylori* (*H. pylori*) infection[12-15]. In HD patients, the role of chronic *H. pylori* infection in their prognosis and quality of life (QOL) has not been defined.

In general, QOL in HD patients is poor. This affects their nutritional status, and thereby contributes to the development of malnutrition, which is a potent predictor of morbidity and mortality[16,17]. The state of metabolic and nutritional derangement called protein-energy wasting (PEW) has a major impact on mortality in HD patients[16,17]. Improving the prognosis of HD patients with PEW requires determination of its etiology and the development of prophylactic strategies[18,19].

The complex interactions of gastroduodenal disease, nutritional status, and *H. pylori* infection in HD patients (which tends to decrease with increasing time on dialysis[20]) remain to be elucidated. Here, we review the association between *H. pylori* infection and HD, and the relationship between *H. pylori* and nutritional status in this population. Finally, we review the effects of *H. pylori* eradication therapy in *H. pylori*-positive HD patients on nutritional status and plasma ghrelin levels.

## *H. PYLORI* INFECTION IN HD PATIENTS

*H. pylori* is a spiral-shaped, microaerophilic Gram-negative flagellate bacterium isolated in 1983 from gastric biopsy specimens of patients with chronic atrophic gastritis[21]. The gastric mucosa of approximately 50% of the world's population is infected with *H. pylori*, and the infection levels exceed 70% in some developing areas[15,22-24].

Previously, we reported that the prevalence of *H. pylori* infection in 539 Japanese HD patients with a mean treatment period of 8.4 ± 0.3 year in 1997 was 48.6% (95%CI: 44.3%-52.9%). This was significantly lower than that in dyspepsia patients with normal renal function [78.5% (74.1%-82.4%), $P < 0.001$] and individuals receiving annual health checks [69.4% (60.3%-77.5%), $P < 0.001$][20]. In a meta-analysis of reports investigating the prevalence of *H. pylori* in dialysis patients before 2009, the prevalence in patients receiving HD and continuous ambulatory peritoneal dialysis (CAPD) was 43.9% [(95%CI: 42.2%-45.6%), 1435/3272] and 34.8% [(29.6%-40.2%), 113/325], respectively, which was again significantly lower than that in individuals with normal renal function [49.8% (48.0%-51.7%), 1476/2961, $P < 0.001$][25]. Although infection rates differ among different geographic populations, in East Asian countries where the prevalence of *H. pylori* infection and incidence of gastric cancer is relatively high, the latest statistics show the infection rate in HD patients to be 44.5% (41.55%-47.6%, 474/1065), which is significantly lower than that in individuals with normal renal function [54.0% (50.9%-57.1%), 560/1038, $P < 0.001$][25]. Importantly, the prevalence in individuals with normal renal function is similar in patients receiving HD treatment for < 1 year[20]. HD treatment, but not uremia from chronic renal failure, may play an important role in the decreased prevalence of *H. pylori* infection.

Recently, infection rates of *H. pylori* have been decreasing. A large-scale Japanese epidemiological study showed that the infection rate in Japanese has declined to 30%-50%, especially in younger patients[26]. Supporting this phenomenon, an investigation of 500 Japanese HD patients with a mean treatment duration of 6.9 ± 6.6 years (2015) reported that the prevalence of infection had dramatically decreased, to 15.0% (95%CI: 12.0-18.4)[27]. Although it has not yet been proven, decreasing rates of *H. pylori* infection suggest

**Table 1 Hemodialysis treatment duration and *Helicobacter pylori* infection status in hemodialysis patients[27], %**

| | < 1 yr | 1-3 yr | 3-10 yr | > 10 yr | *P* value |
|---|---|---|---|---|---|
| *H. pylori* infection rate | 23.8 (15/63) | 16.7 (18/108) | 15.0 (34/226) | 7.8 (8/13) | 0.043 |
| Rate of *H. pylori* negatives | 55.5 (35/63) | 62.0 (67/108) | 68.1 (154/226) | 68.9 (71/103) | > 0.05 |

that the incidence of peptic ulcer disease and gastric cancer is expected to be decreasing in HD patients and that QOL in HD patients has improved due to decreases in *H. pylori*-related gastrointestinal disease.

## TREATMENT PERIODS OF HD AND *H. PYLORI* INFECTION

There is an inverse relationship between *H. pylori* infection and dialysis treatment duration (Table 1)[20,27-31]. We showed that the duration of HD treatment in *H. pylori*-positive patients was 4.6 ± 3.8 years, which is significantly shorter than that in *H. pylori*-negatives (7.3 ± 6.9 years, $P = 0.001$)[27]. Interestingly, the finding of decreased *H. pylori* infection is characteristic of the prevalence of infection decreasing when the treatment period is ⩾ 2 year[28], and the infection rate gradually decreases up to four years after the initiation of HD and is followed by a plateau[20]. In a 4-year follow-up survey of *H. pylori*-positive patients, the prevalence of infection was 51.6% at 1 year, 42.9% at 2 year, and 38.3% at 4 year in the absence of eradication therapy. In other words, 26.7% of patients were naturally cured of *H. pylori* infection over four years[20].

It is unknown why HD patients have a lower prevalence of *H. pylori* infection. One hypothesis is that HD patients have higher levels of pro-inflammatory cytokines[32]. As a result, gastric atrophy progresses, and finally *H. pylori* are not able to live in the gastric mucosa[33-35]. Another hypothesis is that elevated blood urea and urea nitrogen levels may inhibit *H. pylori* growth[36]. A third hypothesis is that *H. pylori* may be cured with incidental antibiotic treatment, because most HD patients suffer from an increased incidence of bacterial infections, and because plasma levels of antimicrobial agents may be higher in HD patients than in individuals with normal renal function[37].

## PEPSINOGEN IN HD PATIENTS

Human pepsinogens (PGs) are proenzymes that act on pepsin. Serum PG levels reflect the status of the gastric mucosa, and decreased PG secretion is a marker of gastric mucosal atrophy. In patients without renal dysfunction, measurements of serum PG levels are used in screening for gastric cancer and gastric mucosal atrophy[38]. In addition, serum PG levels and the PG I/PG II ratio are useful in determining the level of gastric acid secretion[39]. Recently in Japan, a combination of the serum PG level and *H. pylori*-IgG level, namely the ABC method, has been commonly used at health screenings as a useful marker for gastric cancer[40]. Because PG is eliminated *via* the kidney, serum PG levels are elevated in patients with renal dysfunction[41]. The value of serum PG levels as a biomarker of gastric atrophy and the capacity of gastric acid secretion in HD patients was heretofore unknown.

A recent report has demonstrated that PG I and II levels and PG I/PG II ratios in *H. pylori*-negative HD patients are significantly higher than those in *H. pylori*-positives and that PG I levels positively correlate with PG II levels and PG I/PG II ratio in *H. pylori*-negative HD patients ($|R| = 0.849$ and $|R| = 0.569$), past-infection patients ($|R| = 0.870$ and $|R|=0.575$) and current-infection patients ($|R| = 0.784$ and $|R| = 0.517$)[26,42]. In addition, a receiver operating characteristic curve using a cut-off value of 7.75 demonstrated that the sensitivity and specificity of PG I/PG II ratio in predicting the absence of *H. pylori* were 88.7% and 84.0%, respectively[26]. Therefore, serum PG I/PG II ratio may be a valid marker for *H. pylori* infection status and gastric mucosal atrophy in HD patients. Further large-scale studies are needed to verify this.

## NECESSITY OF *H. PYLORI* ERADICATION THERAPY FOR HD PATIENTS

The incidence rates of peptic ulcer and gastric cancer in HD patients are higher than those in individuals with normal renal function[7,43]. In addition, because most HD patients receive anti-thrombotic therapy and/or non-steroidal anti-inflammatory drugs (NSAIDs), the development of drug-induced ulcers and hemorrhage from gastroduodenal lesions easily occurs and often causes fatal blood loss. Therefore, prompt *H. pylori* eradication therapy is necessary for *H. pylori*-infected HD patients[12,13], especially in HD patients with a higher risk of disease development, such as those with a past history of peptic ulcer, gastroduodenal hemorrhage, or use of anticoagulants and/or NSAIDs.

To reduce the risk of gastric cancer, the Japanese health insurance system in 2012 began covering *H. pylori* eradication therapy for all patients with endoscopic gastritis as well for peptic ulcers, gastric mucosa-associated-lymphoid tissue (MALT) lymphoma, post-endoscopic resection of early gastric cancer, and idiopathic thrombocytopenic purpura (ITP)[44-49]. In Japan, first-line eradication therapy is limited to a regimen that employs a standard dose of vonoprazan

or proton pump inhibitor (PPI) administered twice daily, amoxicillin (AMPC) 750 mg twice daily, and clarithromycin (CAM) 200 mg or 400 mg twice daily for 1 wk. Unfortunately, because the prevalence of CAM-resistant *H. pylori* strains in Japan is increasing (> 30%), the eradication rate is gradually decreasing[14,50-53]. Eradication therapy is more challenging in HD patients since they have many exposures to antimicrobial agents due to immune system impairment[54,55]. In fact, 36.4% of patients with chronic renal failure are reported to be infected with CAM-resistant strains, which is significantly higher than in patients with normal renal function (15.2%)[54]. Our recent data published in 2017 shows that rate of CAM-resistant strains in HD patients is 40.5% of infected patients[55]. Alternative regimens may be designed to use *H. pylori*-susceptible antimicrobial agents, increased dosages of antimicrobial agents and PPIs, increased dosing frequency, and longer treatment periods, according to international treatment guidelines[13,52,56-60].

There is no optimal *H. pylori* eradication regimen in HD patients yet (Table 2). Some antimicrobial agents, especially AMPC, are known to exacerbate renal dysfunction. The maximum drug concentration of AMPC in patients with renal failure is 2-4 times higher than in patients with normal renal function, and the half-life is 5-20 times as long as that in healthy individuals[37]. Although several previous reports showed no severe adverse effects of AMPC in HD patients receiving eradication therapy[4,11,30,61-65], the Japanese guidelines for *H. pylori* eradication therapy in the Japanese Society of Helicobacter Research recommends a reduction in AMPC dosage for HD patients[12] and the Japanese drug prescribing guidelines accordingly recommend that the dosage of AMPC for patients with renal failure should be reduced by 70%. In fact, the toxic effects of AMPC in HD patients have been reported in various studies[66-68]; for example, Sheu *et al*[68] reported that patients with a lansoprazole-CAM-metronidazole regimen had a lower risk of acute renal failure than those with a lansoprazole-CAM-AMPC regimen (2% *vs* 18%; relative risk, 0.128, 95%CI: 0.016-0.979) for chronic renal failure non-dialysis patients. Overdose of drugs has to be carefully prevented. Although an optimal regimen for dosage and periods of AMPC in HD patients is not described in the Japanese guidelines for eradication[12], an AMPC-reduced regimen may be appropriate in HD patients.

Recently, we have adopted a regimen composed of PPI and CAM, both at conventional dosage, and a dose of AMPC that is one-third of the conventional dosage (250 mg twice daily), and investigated the efficacy and safety of this regimen[55]. This regimen in HD patients provided equivalent efficacy as the standard dose in conventional therapy for non-dialysis patients in Japan (82.4% and 82.4%, respectively)[55]. Although this suggests that AMPC-reduced triple therapy is effective and safe for HD patients[55,69,70], the sample number of these reports is small, and it is necessary to set an optimal regimen in HD using a larger number of subjects.

Although the eradication rate with the Japanese standard triple therapy was first reported as approximately 85%-91%, it has gradually decreased year by year because of increased prevalence of CAM-resistant strains of *H. pylori*. Because the eradication rates with tailored treatments based only on CAM susceptibility are not very high (71.9%-94.3%), more advanced tailored treatment considering other factors (*e.g.*, different doses of antibiotics and PPIs, different dosages and treatment period) are required to achieve high eradication rates. A tailored *H. pylori* eradication regimen based on CAM susceptibility and maintaining acid secretion (rabeprazole, 10 mg, q.i.d.) is useful because it can achieve an eradication rate exceeding 95%, irrespective of eradication history, thus overcoming differences among CYP2C19 genotypes[52]. However, there was no report to investigate efficacy of tailored regimen in HD patients.

## *H. PYLORI* INFECTION AND NUTRITION STATUS IN HD PATIENTS

HD patients have many risk factors that affect mortality, such as chronic inflammation and metabolic and nutritional derangement[16,17,71]. PEW is defined as a state of decreased body stores of protein and energy fuels (body protein and fat mass) and is diagnosed if three features are present: (1) Abnormal nutrition markers (*i.e.*, low serum levels of albumin, transthyretin or cholesterol); (2) reduced body mass (*i.e.*, low or reduced body or fat mass or weight loss with reduced intake of protein and energy); and (3) reduced muscle mass (*i.e.*, muscle wasting or sarcopenia, and reduced mid-arm muscle circumference)[72].

Ghrelin, an orexigenic peptide released primarily from endocrine cells in the stomach, is important in the pathogenesis of PEW in HD patients[71,73,74]. Ghrelin has multiple functions, including enhancement of the orexigenic effect, protein anabolism, anti-inflammatory action, and cardiovascular protection[74,75,76]. Plasma ghrelin levels increase after fasting and decrease after eating. Ghrelin levels are elevated in patients with a lean body[77]. Plasma ghrelin levels have been found to be associated with malnutrition in patients with advanced-stage cancer and anorexia nervosa[76]. In HD patients, a low ghrelin level increases the risk of cardiovascular mortality and morbidity[78], and the utility of monitoring plasma ghrelin at fixed intervals has been proven as a biomarker for mortality in HD patients[71].

*H. pylori* infections affect ghrelin levels. *H. pylori*-positive patients have lower gastric mucosal and plasma ghrelin levels and a smaller population of ghrelin-positive cells in the gastric mucosa[79]. Although subjects with normal renal function show a correlation between plasma ghrelin level and the severity of gastric mucosal atrophy[79], the association between ghrelin and *H. pylori* infection and between ghrelin and gastric

**Table 2 *Helicobacter pylori* eradication therapy for chronic renal failure patients**

| Year | Author | Country | *n* | Regimen | Treatment period | Eradication rate (%) | Analytic methods |
|---|---|---|---|---|---|---|---|
| 1997 | Tamura *et al*[61] | Japan | 14 | LPZ (30) oid/ 8 wk, AMPC (500) oid/ 3 wk, plaunotol (80) tid/ 24 wk | 21 d | 78.6 | RUT, Culture, Histology |
| 1998 | Munos de Bustillo E *et al*[30] | Spain | 23 | OPZ (20) bid, AMPC (500) tid | 14 d | 60.8 | UBT |
| | | | 23 | plus OPZ (20) bid, CAM (500) bid | 14 d | 82.6 | |
| 1998 | Tokushima *et al*[62] | Japan | 17 | LPZ (30) oid/ 8 wk, AMPC (500) | 21 d | 76.5 | RUT, Culture, Histology |
| | | | 10 | LPZ (30) oid, AMPC (250), MNZ (250) bid/ | 7 d | 90 | |
| 1999 | Araki *et al*[63] | Japan | 17 | OPZ (20) oid/ 8 wk, AMPC (250) oid, CAM (200) oid/ 3 wk, polaprizinc (0.5) bid/ 24 wk | 21 d | 88.2 | IgG, Histology |
| 1999 | Gur *et al*[11] | Turkey | 25 | FAM (40) oid, CAM (500) bid, MNZ (250) bid | 15 d | 80 | Histology, RUT |
| 2001 | Wang *et al*[64] | China | 38 | OPZ (20), AMPC (1000), CAM (500) bid | 7 d | 86.8 | Stool |
| 2002 | Mak *et al*[91] | China | 21 (CRF) | OPZ (20), AMPC (1000), CAM (500) bid | 7 d | 90.5 | RUT |
| 2002 | Tsukada *et al*[65] | Japan | 39 | OPZ (30) bid, AMPC (500) tid, CAM (400) bid | 7 d | 82.1 | UBT |
| 2003 | Mak *et al*[92] | China | 25 (CRF) | OPZ (20) or LPZ (30), AMPC (1000), CAM (500) bid | 7 d | 96 | Histology |
| 2003 | Sheu *et al*[67] | China | 38 (CRF) | LPZ (30), AMPC (750), CAM (500) bid | 7 d | 76.3 | |
| | | | 40 (CRF) | LPZ (30), CAM (500), MNZ (500) bid | 7 d | 92.5 | Stool |
| 2004 | Sezer *et al*[4] | Turkey | 17 | OPZ (20), AMPC (1000), CAM (500) bid/ | 14 d | 94.1 | Endoscopy |
| 2007 | Tseng *et al*[93] | China | 34 (CRF) | ESO (40) or OPZ (20) bid, AMPC (1000) bid, CAM (500) bid | 7 d | 94.1 | UBT |
| 2007 | Itatsu *et al*[69] | Japan | 11 | LPZ (60), AMPC (750), CAM (400) | 7 d | 72.7 | RUT |
| | | | 9 | LPZ (60), CAM (400) | 7 d | 33.3 | |
| 2010 | Change *et al*[70] | Korea | 12 | OPZ (20), AMPC (250), CAM (250), bid | 7 d | 83.4 | RUT, Histology |
| 2010 | Jalalzadeh *et al*[94], Falaknazi *et al*[95] | Iran | 37 | OPZ (20), AMPC (1000), CAM (250), bid | 14 d | 81.1 | IgG, UBT, Stool |
| 2012 | Seyyedmajidi *et al*[96], Jalalzadeh *et al*[97,98] Vafaeimanesh *et al*[99] | Iran | 17 | OPZ (20), AMPC (500), CAM (250), bid | 14 d | 82.4 | UBT, Stool |
| | | | 20 | OPZ (40), AMPC (500), azithromycin (250), bid | 14 d | 80 | |
| 2014 | Makhlough *et al*[100] | Iran | 21 | PPZ (40), AMPC (500), CAM (250), bid | 14 d | 66.7 | RUT, Histology |
| | | | 24 | Sequential therapy (PPT [40] 10 d, AMPC (500) bid, 5 d and CAM (250), tinidazole (500), bid, 5 d | 10 d | 84 | |
| 2016 | Makhlough *et al*[101] | Iran | 20 | PPZ (40), AMPC (500), CAM (500), bid | 14 d | 70 | RUT, Stool |
| | | | 20 | Hybrid regimen PPZ (40), AMPC (500), bid, 7 d + PPZ (40), AMPC (500), CAM (500), tinidazole (500), bid, 7 d | 14 d | 100 | |
| 2018 | Sahara *et al*[55] | Japan | 18 | ESO (20), AMPC (750), CAM (200) bid | 7 d | 77.8 | IgG |
| | | | 19 | ESO (20), AMPC (250), CAM (200) bid | 7 d | 84.2 | |

AMPC: Amoxicillin; CAM: Clarithromycin; ESO: Esomeprazole; FAM: Famotidine; LPZ: Lansoprazole; MNZ: Metronidazole; NA: Not available; OPZ: Omeprazole; PPZ: Pantoprazole; RUT: Rapid urease test; UBT: Urea breath test; bid: Twice-daily dosing; tid: Three-times-daily dosing.

mucosal atrophy in HD patients is less well understood. In an analysis using 78 HD patients and 51 non-dialysis patients with chronic renal disease, des-acyl ghrelin levels in HD patients were significantly higher than those in non-dialysis patients, and ghrelin levels decreased with the progress of endoscopic gastric mucosal atrophy in HD patients (Table 3)[19]. Importantly, acyl-ghrelin levels in the non-*H. pylori* infection HD group (39.4 ± 23.0 fmol/mL) were significantly higher than in patients with current (24.6 ± 17.5 fmol/mL, $P = 0.022$) and past *H. pylori* infection (23.4 ± 19.9 fmol/mL, $P = 0.007$) (Table 4)[18], suggesting that the severity of serological and endoscopic gastric mucosal atrophy is a major determinant of ghrelin levels (Table 3). In fact, multiple regression analysis shows a significant positive correlation between acyl ghrelin and PG I levels ($\beta = 0.738$, $P < 0.001$) and significant negative correlations between ghrelin and age, albumin, and creatinine levels[19]. Therefore, PG level is the most influential determinant of plasma acyl and des-acyl ghrelin levels in HD patients. This suggests that plasma and gastric mucosal ghrelin levels are influenced by not only long-standing enhanced gastric mucosal inflammation induced by *H. pylori* infection but also by gastric mucosal atrophy[79]. Because plasma and gastric ghrelin levels depend on the number of ghrelin immunoreactive cells in the gastric mucosa[79-81], plasma ghrelin levels may be influenced more by the severity of atrophy than current *H. pylori* infection in HD patients. Therefore, it is important to consider methods to prevent progression of gastric mucosal atrophy in HD patients.

HD patients with gastric mucosal atrophy have a lower normalized protein catabolic rate (nPCR) than non-atrophy patients[19]. Chronic persistent damage to the gastric mucosa and gastric mucosal atrophy in *H. pylori*-positive HD patients may contribute to

**Table 3 Clinical characteristics in hemodialysis patients between patients with and without gastric mucosal atrophy[19]**

| | Atrophy (-) | Atrophy (+) | *P*-value |
|---|---|---|---|
| N (Male/Female) | 28 (17/11) | 50 (33/17) | - |
| Age | 67.7 ± 12.3 | 71.6 ± 11.0 | 0.155 |
| Dialysis periods (yr) | 7.5 (2.4-16.8) | 7.7 (3.1-12.7) | 0.681 |
| Acyl-ghrelin | 38.0 (23.5-57.0) | 18.0 (12.0-26.3) | < 0.001 |
| Desacyl-ghrelin | 303 (248-533) | 200 (137-277) | < 0.001 |
| BMI (kg/m$^2$) | 19.8 ± 3.2 | 19.6 ± 2.8 | 0.773 |
| Albumin (g/dL) | 3.5 ± 0.3 | 3.4 ± 0.4 | 0.273 |
| Total cholesterol (mg/dL) | 166 ± 37 | 154 ± 36 | 0.165 |
| Cholinesterase (U/L) | 245 ± 111 | 219 ± 68.7 | 0.205 |
| Intact PTH (pg/mL) | 351 ± 294 | 247 ± 192 | 0.062 |
| Ferritin (ng/mL) | 128 ± 118 | 128 ± 221 | 0.989 |
| PG Ⅰ (ng/mL) | 416.2 (314.2-783.7) | 196.0 (73.8-358.8) | < 0.001 |
| PG Ⅱ (ng/mL) | 42.3 (31.6-60.0) | 28.4 (16.8-45.7) | 0.003 |
| PG Ⅰ / Ⅱ ratio | 10.89 (9.11-13.38) | 7.31 (4.17-11.08) | 0.001 |
| nPCR (g/kg/d) | 0.94 ± 0.14 | 0.85 ± 0.16 | 0.022 |

BMI: Body mass index; BUN: Blood urea nitrogen; PTH: Parathyroid hormone; CRP: C-reactive protein; PG: Pepsinogen; ABI: Ankle-brachial pressure index; nPCR: Normalized protein catabolic rate.

**Table 4 Plasma acyl-ghrelin and desacyl-ghrelin levels according to *Helicobacter pylori* status in hemodialysis patients[18]**

| | Non-infection ($n$ = 29) | Past-infection ($n$ = 27) | Present infection ($n$ = 17) |
|---|---|---|---|
| Plasma acyl-ghrelin (fmol/mL) | 39.4 ± 23.0 | 23.4 ± 19.9[a] | 24.6 ± 17.5[a] |
| Plasma desacyl-ghrelin (fmol/mL) | 353.2 ± 190.2 | 242.1 ± 139.6[a] | 236.3 ± 143.6[a] |

[a]$P < 0.05$ *vs* Non-infection group.

decreased protein intake, PEW, and decreased body weight *via* decreased ghrelin production. Because ghrelin level is associated with mortality related to cardiovascular disease and PEW in HD patients, alternative management, such as *H. pylori* eradication therapy, before the progression of gastric mucosal atrophy might be necessary to prevent the decrease in ghrelin level in HD patients[74,78].

## *H. PYLORI* ERADICATION THERAPY AND NUTRITION STATUS IN HD PATIENTS

*H. pylori* infection affects the incident rate of gastroduodenal disease and nutritional status[20,25]. *H. pylori* eradication therapy often causes individuals with normal renal function to develop hyperlipidemia and hyperproteinaemia, along with an increase of body weight and BMI[80]. This phenomenon is considered to be due to increases in plasma ghrelin level followed by increased appetite and food intake after *H. pylori* eradication therapy[82,83] In CAPD patients, *H. pylori* eradication therapy significantly improves anorexia, inflammation, and malnutrition[84]. After *H. pylori* eradication, CAPD patients with anorexia showed a significant increase in markers of nutrition and in VAS scores for almost all questions. Significant differences were also found in lymphocyte count, nPCR, prealbumin, albumin, CRP, before-lunch desire to eat, after-lunch desire to eat, hunger before lunch, hunger after lunch, fullness before lunch, consumption after lunch, and palatability[85]. However, it is unclear whether nutritional disorders in HD patients improve after eradication therapy. It is important to answer this clinical question to improve the poor prognosis in HD patients.

At 1 year after eradication therapy, serum cholinesterase levels significantly increase compared with the level before eradication (303.2 ± 76.0 *vs* 287.3 ± 68.1 IU/L, $P = 0.029$). In particular, cholesterol (before, 196.6 ± 23.2 mg/dL; after, 206.1 ± 25.9 mg/dL, $P = 0.042$) and cholinesterase levels (before, 296.9 ± 70.8 IU/L; after, 316.4 ± 73.8 IU/L, $P = 0.049$) increase more in patients with mild-moderate gastric mucosal atrophy than in those with severe atrophy. This observation suggests that eradication therapy has contributed to improvement of PEW in HD patients. We therefore recommend that HD patients be checked for *H. pylori* infection and that eradication therapy should be initiated before the progression of gastric atrophy.

It is possible that the improvement in nutritional status and increase in BMI after eradication therapy depends not only on an increase in ghrelin levels but also on another biological mechanism(s), such as an improvement in gastrointestinal motility[86], change in gut microbiome profile[87], and/or increase in absorption ability[88]. Betrapally *et al*[87] reported that alterations to the intestinal microbiota affect the development of nonalcoholic steatohepatitis by influencing digestion, development of obesity, immune response, and production of gut hormones. *H. pylori* eradication therapy changes the gastrointestinal microbiota[89]. A study to examine

whether the long-term prognosis of HD patients is improved by the effect of eradication therapy on the microbiota will be required to investigate this hypothesis further.

## *H. PYLORI* AND ANEMIA IN HEMODIALYSIS PATIENTS

*H pylori* has been identified as a possible cause of vitamin B12 and iron deficiency in the general population. Trimarchi *et al*[90] reported that *H. pylori*-positive HD patients may present with lower vitamin B12 blood levels and that *H. pylori* should be suspected in HD patients when low or low-normal vitamin B12 levels or macrocytosis exist.

## CONCLUSION

Chronic renal failure patients receiving HD have a low prevalence of *H. pylori* infection. More than one-third of patients receiving < 4 year of dialysis had naturally cured *H. pylori* infection within the 4 year observation period. However, because chronic renal failure patients have a higher risk of gastroduodenal disorders, all HD patients are recommended to receive endoscopic check-ups to reduce the chance of developing peptic ulcer disease. Moreover, patients with *H. pylori* infection should also receive eradication therapy including AMPC 250 mg twice daily to prevent peptic ulcer, gastric cancer, and hemorrhage from gastroduodenal lesions. QOL in HD patients is usually poor and affects their nutritional status. Severity of gastric atrophy is shown to be the major determinant of ghrelin levels in HD patients and eradication treatment of *H. pylori* improves nutrition status by increasing serum cholinesterase and cholesterol levels. *H. pylori* eradication before progress of gastric atrophy may be required to prevent a decrease in ghrelin levels and improve prognosis of HD patients in relation to poor nutritional status.

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**P- Reviewer**: Papamichail K, Paoluzi OA, Pellicano R, Slomiany BL
**S- Editor**: Wang XJ **L- Editor**: A **E- Editor**: Huang Y

World Journal of Gastroenterology

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*World J Gastroenterol* 2018 April 21; 24(15): 1601-1615

DOI: 10.3748/wjg.v24.i15.1601

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

MINIREVIEWS

# Multiomics biomarkers for the prediction of nonalcoholic fatty liver disease severity

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Author contributions: All authors equally contributed to this paper with conception and design of the study, literature review and analysis, drafting and critical revision and editing, and final approval of the final version.

Supported by Agencia Nacional de Promoción Científicay Tecnológica, No. PICT 2014-0432, No. PICT 2014-1816 and No. PICT 2015-0551.

Conflict-of-interest statement: No potential conflicts of interest.



Manuscript source: Invited manuscript

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Received: January 1, 2018
Peer-review started: February 1, 2018
First decision: February 24, 2018
Revised: March 13, 2018
Accepted: March 31, 2018
Article in press: March 31, 2018
Published online: April 21, 2018

## Abstract

This review intends to uncover how information from large-scale genetic profiling (whole genome sequencing, and whole exome sequencing) of nonalcoholic fatty liver disease (NAFLD), as well as information from circulating transcriptomics (cell-free miRNAs) and metabolomics, contributes to the understanding of NAFLD pathogenesis. A further aim is to address the question of whether OMICs information is ready to be implemented in the clinics. The available evidence suggests that any new knowledge pertaining to molecular signatures associated with NAFLD and nonalcoholic steatohepatitis should be promptly translated into the clinical setting. Nevertheless, rigorous steps that must include validation and replication are mandatory before utilizing OMICs biomarkers in diagnostics to identify patients at risk of advanced disease, including liver cancer.

**Key words:** Nonalcoholic steatohepatitis; Fibrosis; Liver biopsy; Genetics; *PNPLA3*; *TM6SF2*; Metabolomics; Proteomics; Transcriptomics; Nonalcoholic fatty liver disease; miR122

**Core tip:** It is expected that, in the near future, nonalcoholic fatty liver disease patients can be diagnosed and treated according to their own "molecular signature". Specific focus should be placed on prevention and early diagnosis through the application of biomarkers of disease risk. Selection of "personalized drugs" as well as tailored therapy according to the specific molecular signature should be further guaranteed.

Pirola CJ, Sookoian S. Multiomics biomarkers for the prediction of nonalcoholic fatty liver disease severity. *World J Gastroenterol* 2018; 24(15): 1601-1615 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i15/1601.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i15.1601

## INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a chronic liver disease that affects adult and children populations around the world, with prevalence reaching alarming levels[1,2].

NAFLD may progress from a benign histological disease stage characterized by plain fat accumulation, usually referred to as simple steatosis or nonalcoholic fatty liver (NAFL), to a more severe histological form characterized by liver cell injury, a mixed inflammatory lobular infiltrate, and variable fibrosis named nonalcoholic steatohepatitis (NASH)[3,4].

Precise histological diagnosis, including disease stages (NAFL and NASH), is commonly based on liver biopsy[2]. Nevertheless, because this method imposes certain limitations, including potential complications such as bleeding and patients' abdominal discomfort, and needs to be performed in a special setting, noninvasive approaches are favored and have gained considerable attention. It is also noteworthy that the histological diagnosis of the severity of NAFLD might be potentially biased if a small portion of hepatic tissue is sampled.

Hence, significant clinical and research efforts are currently being directed toward the search for reliable biomarkers aimed at the prediction of the disease severity and prognosis.

Knowledge in the field of liver diseases, particularly NAFLD, has benefitted in the last ten years from the rapid development of high-throughput technologies, including genomics, transcriptomics, proteomics and metabolomics. This review intends to uncover how information from large-scale genetic profiling (whole genome sequencing and whole exome sequencing) of NAFLD, as well as information from transcriptomics and metabolomics, and the interplay of these personal characteristics with dietary factors may contribute to the diagnosis and risk prediction of NAFLD progression. In addition, the question of whether OMICs information is ready to be implemented in the clinics will be addressed.

A brief description of OMICs signatures, including their main applications as biomarkers in clinical practice, is provided in Figure 1. OMICs biomarkers may be considered either for screening purposes to assess the disease risk or exposure, or for the assessment of the disease severity and prognosis, and/or for monitoring treatment response (Figure 1).

## ROLE OF GENETIC MARKERS IN THE PREDICTION OF NAFLD RISK AND DISEASE SEVERITY

Although the pathogenesis of NAFLD is not understood fully, a growing body of evidence indicates that the disease develops from a complex process involving many factors, including genetic susceptibility and environmental insults[5,6].

In fact, the results yielded by the first genome-wide association study on NAFLD[7] on the role of rs738409 C/G -a variant nonsynonymous single nucleotide polymorphism (SNP) of *PNPLA3* (patatin-like phospholipase domain containing 3, also known as adiponutrin or calcium-independent phospholipase A2-epsilon) have significantly contributed to the knowledge of the genetic component of NAFLD. This finding was subsequently widely replicated around the world, confirming that the G allele in the forward strand is significantly associated not only with an increased risk of fatty liver but the histological disease severity as well[8,9] (OR 1.88 per G allele). In fact, rs738409 explains about 5.3% of the total variance in NAFLD[9].

Furthermore, results of the first exome-wide association study of liver fat content indicate that rs58542926 (E167K), a nonsynonymous variant located in *TM6SF2* (Transmembrane 6 Superfamily Member 2), is significantly associated with increased liver fat content[10]. Nevertheless, in contrast to the effect of the variant located in *PNPLA3*, the rs58542926 exerts a moderate effect on the risk of NAFLD (odds ratio: 2.13)[11]. Subsequent studies have also revealed an association of rs58542926 with the disease severity[12-14], as well as dual and opposite role in cardiovascular disease prevention[11,12,15].

Thus, it is reasonable to speculate that genetic markers, particularly the 738409-G risk allele, may be used for individual risk assessment either alone or as a part of multi-score biomarkers (Figure 2). For example, Kotronen and coworkers evaluated the performance of rs738409 in predicting the risk of NAFLD by combining routine clinical and laboratory data and the rs738409 genotypes[16]. The authors observed a sensitivity of 86% and a specificity of 71% in the estimation of increased liver fat content[16]. Surprisingly, addition of the genetic information to the score improved the accuracy of NAFLD prediction by less than 1%.

The incorporation of genetic markers into noni-

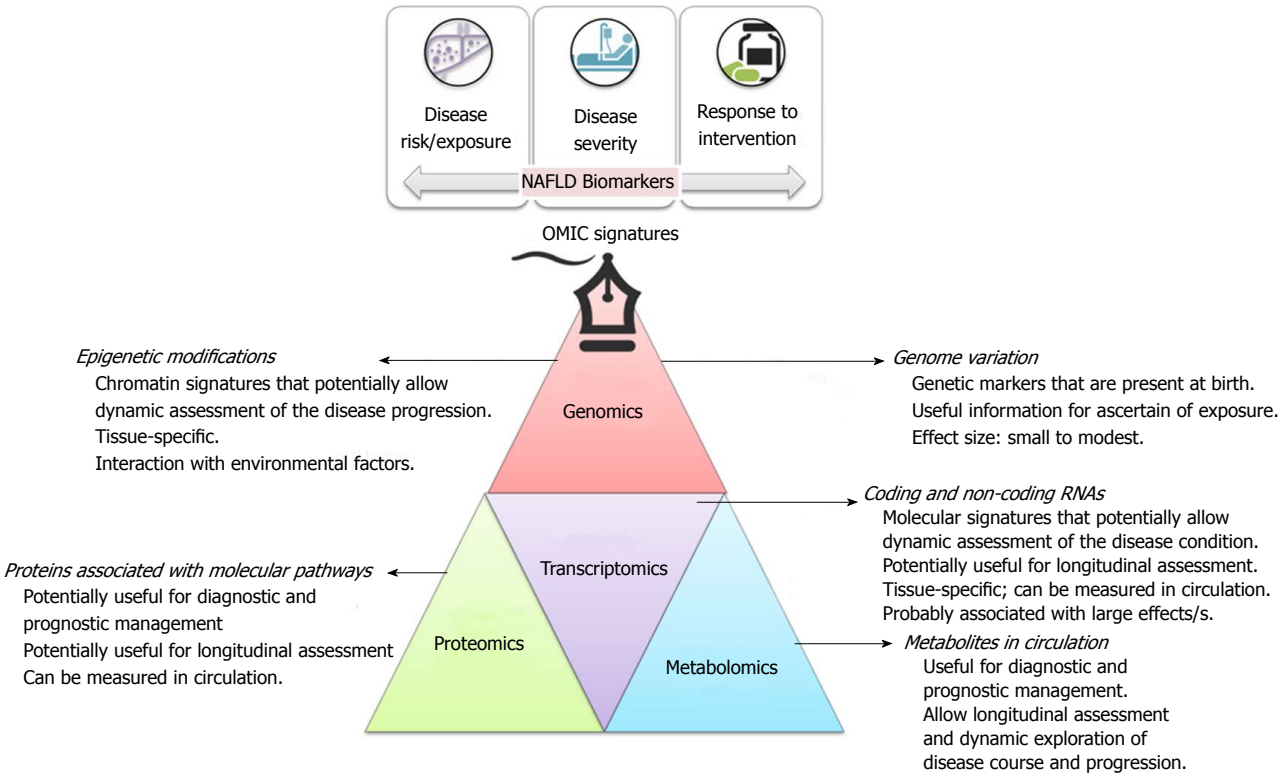



**Figure 1 Brief description of OMICs signatures, including their main applications as biomarkers in clinical practice.**

nvasive tests that discriminate between NAFL and NASH results in a more challenging strategy; despite these difficulties, there have been some interesting attempts. For instance, a risk score comprising of both clinical and genetic (*PNPLA3* rs738409 C>G, *SOD2* rs4880 C>T, *KLF6* rs3750861 G>A, and *LPIN1* rs13412852 C>T) risk factors resulted in an AUROC (Area Under the Receiver Operating Characteristic) of 0.80 to predict NASH in obese children with increased levels of liver enzymes[17], as shown in Figure 2.

Other examples include the *NASH Clin Score* that combines laboratory tests (AST, fasting insulin) and rs738409 genotypes, and the NASH ClinLipMet Score that combines laboratory test (AST, fasting insulin), circulating metabolites (glutamate, isoleucine, glycine, lyso PC 16:0; PE 40:6) and rs738409 genotypes[18], as depicted in Figure 2.

Furthermore, promising results have been reported on the use of genetic markers in predicting NAFLD-intervention response, as summarized in Figure 2. For example, it was observed that genetic variation in *PNPLA3* might confer sensitivity to liver fat content decrease in obese patients undergoing weight loss[19]. The findings yielded by this study, though based on a small number of subjects, suggested that weight loss was more effective in decreasing liver fat in subjects who were homozygous for the rs738409-G allele[19]. Likewise, rs738409 correlated with changes in metabolic profile and intrahepatic triglyceride content (IHTG) as measured by proton magnetic resonance spectroscopy in patients enrolled in a lifestyle modification program[20]. Concordant results were reported regarding greater improvement in hepatic steatosis after bariatric surgery in the risk-G-rs738409 allele carriers[21] (Figure 2).

A different approach to the use of genetic testing based on single base variations in the DNA sequence requires search for variants in mitochondrial DNA (mtDNA). Mitochondria contain their own genetic information in the mtDNA (16.5 kb), which is maternally inherited; the 13 mtDNA-encoded proteins are all components of the oxidative phosphorylation (OXPHOS). A comprehensive exploration of the complete liver mtDNA-mutation spectrum in patients with NAFLD during different stages of the disease by next generation sequencing showed that the disease severity is associated with an increased liver mtDNA mutational burden, including point mutations in OXPHOS-genes that showed high degrees of heteroplasmy[22]. Given that the variability in the mt-genomes observed in NAFLD and NASH seems to originate from a common germline source, rather than from tissue-specific mutations, point mutations can also be assessed in samples of peripheral blood mononuclear cells[22].

## ROLE OF EPIGENETIC MODIFICATIONS AS NONINVASIVE BIOMARKERS OF NAFLD AND NASH

The dynamic nature of epigenetic modifications is not only an ideal frame to explain the cross-talk between NAFLD and related phenotypes, including

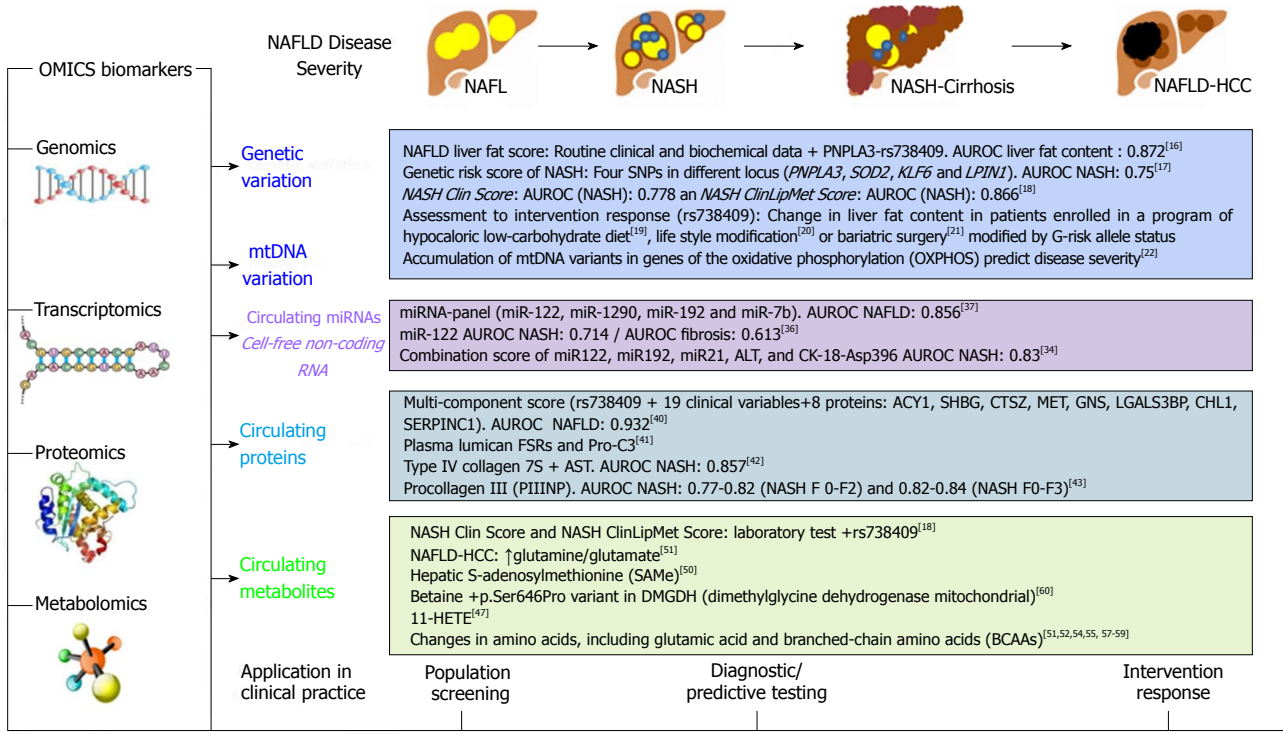



**Figure 2 Summary of OMICs biomarkers in the prediction of nonalcoholic fatty liver disease severity.**

insulin resistance[23], but is also an attractive target for therapeutic intervention[24]. Treatment-induced epigenetic remodeling of liver tissue was observed in a cohort of obese patients with NAFLD who underwent bariatric surgery[24]. In addition, changes in DNA methylation could be used as a target of a biomarker that allows monitoring, for instance, effectiveness of pharmacotherapy. Interesting results have been reported in the context of other non-cancer complex diseases, including rheumatoid arthritis[25], pediatric asthma[26] or anxiety disorders[27].

It is worth noting that epigenetic modifications, *i.e.* DNA methylation, are not restricted to the nuclear genome, but can also be found in mt-genomes[28]. In fact, we found for the first time that hepatic methylation and transcriptional activity of the MT-ND6 (mt genome-encoded NADH deshydrogenase 6, a member of the OXPHOS complex 1) are associated with the histological severity of NAFLD[29]. This epigenetic change to mtDNA is potentially reversible by lifestyle interventional programs, as physical activity could modulate the methylation status of MT-ND6[29].

## CELL-FREE DNA AND RNA AS NONINVASIVE BIOMARKERS OF NASH

Circulating molecular biomarkers, particularly cell-free DNA (cfDNA) and cell-free RNA (cfRNA) are focus of intensive research; however, the strategies employed in these studies are not necessarily novel. In fact, the first description of cell-free nucleic acids (cfNAs) was provided by Mandel and Métais in 1948[30]; indeed, these authors introduced the concept of liquid biopsy.

Basically, cfNAs refer to molecules of nucleic acids that circulate free of cells in the bloodstream and the source of which is primarily dying cells from distant tissues.

Considerable efforts have been dedicated to the use cfDNA for the prediction of liver fibrosis associated with NASH and alcoholic liver disease[31]; however, the preliminary results indicate substantial lack of specificity, as they can be completely unrelated to NASH-biology[32]. Furthermore, the fact that cfDNA circulates not only at very low concentrations but is also highly fragmented imposes analytical and technical challenges that are very difficult to overcome[33].

Conversely, detection of microRNAs (miRNAs), which are highly conserved noncoding small RNAs, has demonstrated quite robust performance, particularly in the circulating compartment. In addition, unlike cfDNA, cfmiRNAs are resistant to degradation as well as to several freeze–thaw cycles, making them ideal biomarkers for use in the clinical setting.

The circulating miRNA signature of NAFLD has been extensively explored in case-control studies, including patients with liver biopsy[34-37], Figure 2. Studies in which liver and circulating miRNA levels were compared demonstrated that cfmiRNAs are good predictors of NAFLD-disease stages[36]. Specifically, circulating miR122 and miR192 not only mirror histological and molecular events occurring in the liver, but have a reliable predictive power in differentiating simple steatosis from NASH[36]. Thus, it can be posited

that cfmiRNAs are reliable candidates for incorporation into multi-panel scores for the prediction of NAFLD and NASH (Figure 2).

For example, a miRNA panel, composed by the detection of miR122-5p, miR1290, miR27b-3p, and miR192-5p) showed a high diagnostic accuracy for NAFLD[37] (Figure 2). A combination score that included miR122, miR192, miR21, ALT, and CK-18-Asp396 exhibited an AUROC of 0.83 for the prediction of NASH[34] (Figure 2).

## ROLE OF CIRCULATING PROTEINS IN THE PREDICTION OF NASH SEVERITY

The use of proteins that circulate in serum or plasma for predicting liver-related histological outcomes, specifically liver fibrosis, has been largely relegated probably because such approaches are technically challenging, while offering low performance and poor accuracy. The most remarkable example of this strategy is based on the use of plasma caspase-generated cytokeratin-18 fragments (CK-18) as a noninvasive alternative biomarker of NASH. Results from a large multicenter study showed that plasma CK-18 has relatively good specificity for NAFLD (AUROC: 0.77), NASH (0.65) and fibrosis (0.68). Nevertheless, the overall sensitivity for NAFLD (63%), NASH (58%) and fibrosis (54%) is limited, making this test inadequate for use as a single noninvasive screening test[38].

Interesting attempts to develop multi-component tests that integrate clinical and laboratory data, including circulating proteins, have also been made. For example, we have tested a diagnostic model based on a composite index using clinical and laboratory data, including circulating biomarkers such as soluble intercellular adhesion molecule-1 (sICAM-1), which was able to differentiate between patients with simple steatosis and NASH with a post-test probability for NASH of 99.5% when all positive tests were present[39].

There are similar proposals - though restricted to the prediction of NAFLD but not NASH - based on OMICs-derived data, including genetic information (rs738409), clinical variables, and measurement of different proteins (ACY1, SHBG, CTSZ, MET, GNS, LGALS3BP, CHL1, SERPINC1), which - if combined - seem to be quite reliable in disease risk identification (AUROC for steatosis 0.935)[40]. Nevertheless, it seems that this approach has limited cost-effectiveness for NAFLD-screening programs.

Latest advancements in this field focus directly on disease phenotypes, for example liver fibrosis, which target the detection of excess collagen synthesis rate both directly in liver tissue and noninvasively in blood[41].

The combination of type IV collagen 7S and aspartate aminotransferase (AST) in a multi-test for the prediction of NASH-fibrosis showed promising results[42]. Likewise, measurement of circulating procollagen III (PIIINP) has been quite accurate in the prediction of NASH (AUROC 0.77-0.82) and NASH-fibrosis (0.82-0.84)[43].

Unfortunately, proteomic analysis using state of the art technology is currently poorly developed in the field of NAFLD. In fact, robust attempts to refine, replicate and follow-up on putative discovered proteins have not been done, even though some promising studies have been carried out. For example, using MALDI TOF/TOF and western blot analysis of coupled tissue and serum samples allowed the identification of two interesting protein candidates, including the mitochondrial enzyme CPS1 (Carbamoyl-Phosphate Synthase 1) and GRP78, also known as heat shock protein family A (Hsp70) member 5, which could stratify the different phenotypes associated with the disease severity[44]. Results obtained by using similar approaches, including SELDI-TOF mass spectrometry[45] and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI TOF-MS)[46] have been published. Still, the identified peaks require validation, replication and large-scale testing.

## CIRCULATING METABOLITES IN NASH PREDICTION

Initial case-control studies on plasma metabolomics of NAFLD have been performed years ago by Puri *et al*[47], who conducted a comprehensive analysis of plasma lipids and eicosanoid metabolites quantified by mass spectrometry. The authors reported a stepwise increase in lipoxygenase (LOX) metabolites, 5(S)-hydroxyeicosatetraenoic acid (5-HETE), 8-HETE and 15-HETE that characterized the progression from normal liver to NAFL to NASH[47]. Puri and colleagues found that the level of 11-HETE, a nonenzymatic oxidation product of arachidonic (20:4) acid, was significantly and specifically increased in NASH but not in NAFL patients[47]. Subsequent studies that included untargeted global metabolomic analysis revealed marked changes in bile salts and glutathione-related metabolites, as well as higher levels of branched-chain amino acids, phosphocholine, carbohydrates (glucose, mannose), lactate and pyruvate, in subjects with severe NAFLD[48]. Regarding bile salts, a recent study indicated that total conjugated primary bile acids were significantly higher in NASH[49].

A novel study in which the authors combined metabolomic data from experimental animals and human samples introduced the interesting concept that NASH might be sub-classified into two major subtypes according to the circulating pattern of triglycerides, diglycerides, fatty acids, ceramides and oxidized fatty acids[50].

As mentioned earlier, interesting strategies that combine clinical, genetic and lipidomic-derived variables into a multi-score have shown good predictive values in differentiating NAFL from NASH. Specifically, Zhou and coworkers reported on the performance of the *NASH*

**Table 1 List of pathways involved in nonalcoholic fatty liver disease selected from significant *Q*-values that dependent on both genes and metabolites analyzed jointly**

| Pathway name | Q-joint |
|---|---|
| Solute carriers -mediated transmembrane transport | 1.23E-12 |
| Transmembrane transport of small molecules | 9.66E-12 |
| Transport of glucose and other sugars bile salts and organic acids metal ions and amine compounds | 8.40E-10 |
| Leukotriene biosynthesis | 8.71E-10 |
| Transport of glucose and other sugars bile salts and organic acids metal ions and amine compounds | 1.91E-09 |
| Transport of inorganic cations-anions and amino acids-oligopeptides | 4.27E-09 |
| Amino acid and oligopeptide SLC transporters | 1.10E-08 |
| Transport of inorganic cations/anions and amino acids/oligopeptides | 2.40E-08 |
| tRNA Aminoacylation | 3.03E-08 |
| Gamma-glutamyl cycle | 3.61E-08 |
| tRNA charging | 5.96E-08 |
| mRNA protein and metabolite induction pathway by cyclosporine A | 8.47E-08 |
| Class I MHC mediated antigen processing & presentation | 1.73E-07 |
| $Na^+/Cl^-$ dependent neurotransmitter transporters | 3.10E-07 |
| Amino acid transport across the plasma membrane | 3.72E-07 |
| S-methyl-5-thio-alpha;-D-ribose 1-phosphate degradation | 6.17E-07 |
| Amine compound solute carrier transporters | 6.17E-07 |
| Protein digestion and absorption - homo sapiens (human) | 2.13E-06 |
| Amino acid interconversion | 2.21E-06 |
| Biochemical pathways part I | 2.34E-06 |
| Amino acid metabolism | 3.96E-06 |
| Aminoacyl-tRNA biosynthesis - homo sapiens (human) | 6.88E-06 |
| Metabolism of amino acids and derivatives | 8.72E-06 |
| Mineral absorption - homo sapiens (human) | 1.47E-05 |
| Cytosolic tRNA aminoacylation | 2.86E-05 |
| Mitochondrial tRNA aminoacylation | 2.86E-05 |
| tRNA Aminoacylation | 2.86E-05 |
| Histidine, lysine, phenylalanine, tyrosine, proline and tryptophan catabolism | 0.000159 |
| Gene expression | 0.000181 |
| Tryptophan catabolism | 0.000275 |
| Phase Ⅱ conjugation | 0.000426 |
| Phenylalanine and tyrosine catabolism | 0.003 |
| Glutamine and glutamate metabolism - homo sapiens (human) | 0.00376 |
| Glutaminolysis and cancer | 0.00493 |
| Glycine metabolism | 0.0052 |
| Glutamate glutamine metabolism | 0.00665 |
| Recycling of bile acids and salts | 0.00669 |
| Glycine serine alanine and threonine metabolism | 0.0101 |
| Branched-chain amino acid catabolism | 0.0103 |

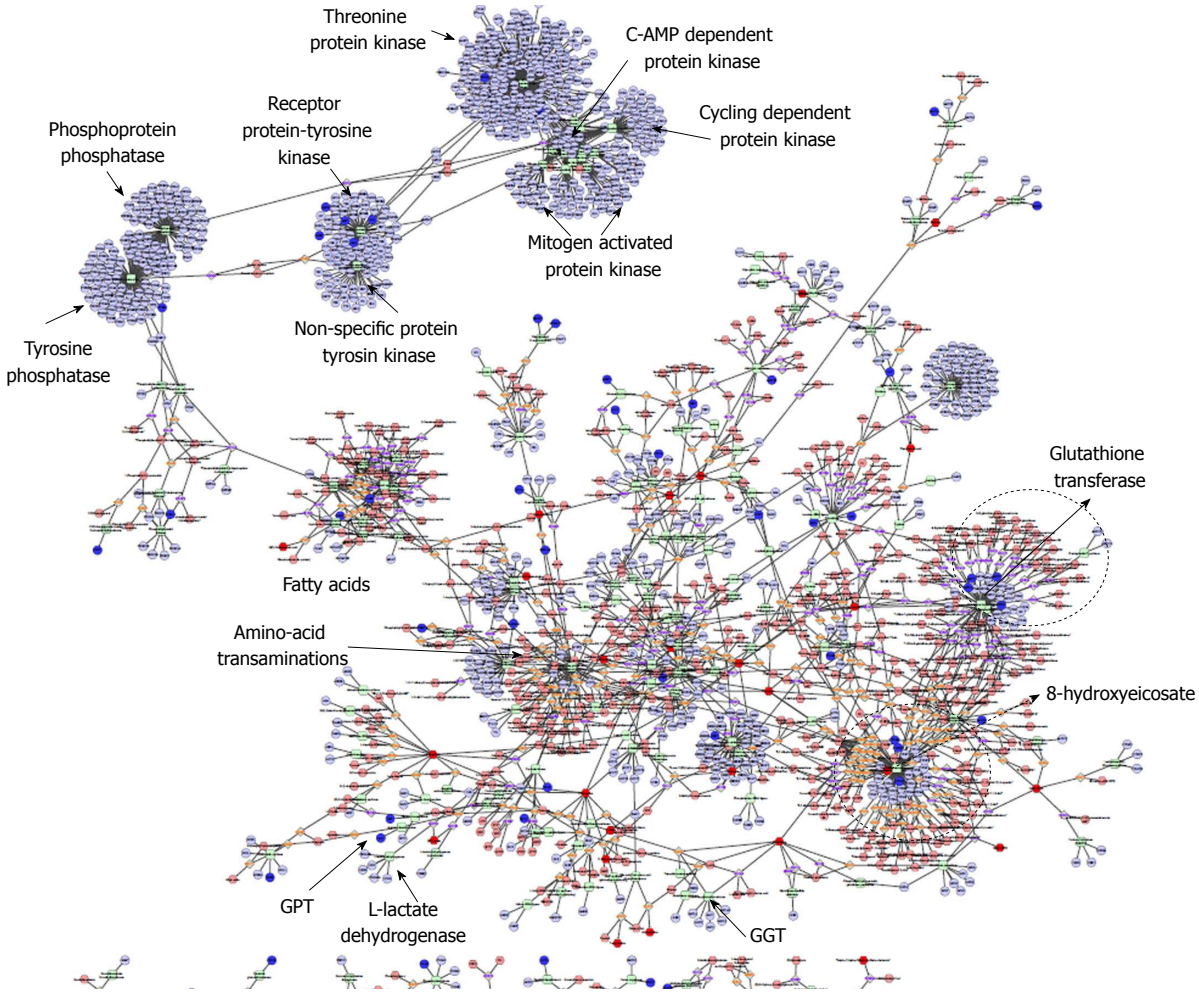
OMICs-integrative analysis was performed using the IMPaLA (integrated molecular pathway level analysis, http://impala.molgen.mpg.de)[67] platform. A joined adjusted *P*-value (*Q*-value) was calculated to control for multiple testing by false discovery rate.

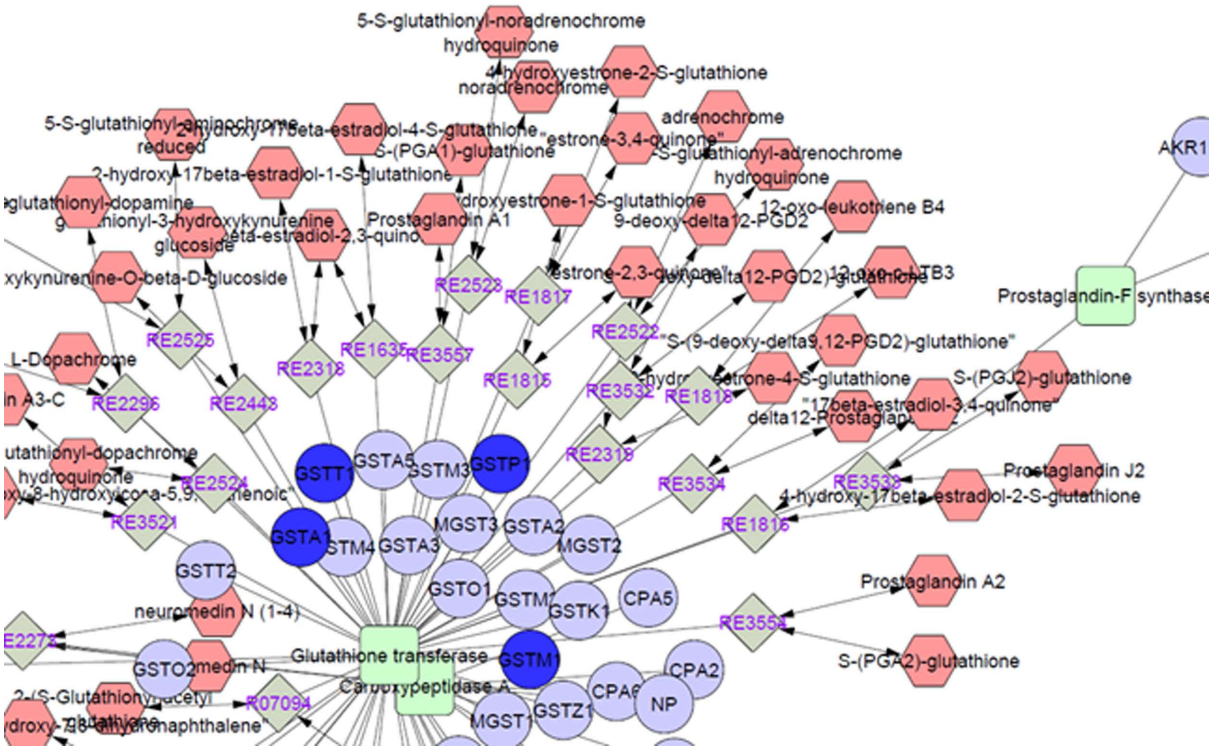
*Clin Score*, obtained through backward stepwise logistic regression analyses of biochemical variables (glutamate, isoleucine, glycine, lysophosphatidylcholine 16:0, phosphoethanolamine 40:6, AST, and fasting insulin), along with rs738409 genotypes[18]; this score identified patients with NASH with an AUROC of 0.866 (Figure 2).

Recent explorations on changes in liver metabolism during NASH development[51,52], along with the findings from high-throughput circulating profiling of patients with metabolic syndrome[53] suggest that elevated levels of alanine (ALT) and aspartate (AST) aminotransaminases in patients with NAFLD are the consequence of impaired liver metabolism of amino acids, including glutamate and aromatic amino acids, rather than a mere biomarker of liver injury[14,52,54]. This observation is consistent with the fact that NASH is associated with changes in the level of circulating amino acids[55], including L-glutamic acid, 2-hydroxyglutarate and alanine / pyruvate ratio, which are significantly associated with NAFLD-disease severity[52,56]. Changes in the level of branched-chain amino acids were described in pediatric population[57], and these findings were replicated in studies on adults as well[58].

Interestingly, alterations in multiple aminoacids, gamma-glutamyl dipeptides and lipids may be related to common genetic variations associated with NAFLD, as observed in earlier *in vitro* studies based on knocking down or over-expression of the pIle148Met (rs738409) isoforms[59].

Finally, a two-stage multicenter case-control study that combined results of NAFLD-histological variables, levels of circulating metabolites and genetic markers indicated that NASH is associated with decreased levels of betaine in circulation. Furthermore, the disease severity is associated with genotypes of the missense variant p.Ser646Pro (rs1805074) in *DMGDH* gene,


Threonine protein kinase
C-AMP dependent protein kinase
Receptor protein-tyrosine kinase
Cycling dependent protein kinase
Phosphoprotein phosphatase
Mitogen activated protein kinase
Non-specific protein tyrosin kinase
Tyrosine phosphatase
Glutathione transferase
Fatty acids
Amino-acid transaminations
8-hydroxyeicosate
GPT
L-lactate dehydrogenase
GGT


5-S-glutathionyl-noradrenochrome hydroquinone
4-hydroxyestrone-2-S-glutathione
noradrenochrome
adrenochrome
2-hydroxy-17beta-estradiol-4-S-glutathione
S-(PGA1)-glutathione
2-hydroxy-17beta-estradiol-1-S-glutathione
S-glutathionyl-adrenochrome hydroquinone
Prostaglandin A1
9-deoxy-delta12-PGD2
12-oxo-leukotriene B4
glucoside
RE2523
RE1817
RE2522
RE2525
RE1635
RE3557
RE2318
RE1815
RE3532
RE1818
RE2296
RE2443
RE2319
RE3534
RE2524
RE3521
RE1816
GSTT1
GSTA5
GSTM3
GSTP1
GSTA1
GSTM4
GSTA3
MGST3
GSTA2
MGST2
GSTT2
GSTO1
GSTM2
GSTK1
CPA5
neuromedin N (1-4)
RE3554
Glutathione transferase
GSTM1
GSTO2
medin N
CPA2
CPA6
NP
GSTZ1
MGST1
R07094
AKR1
Prostaglandin-F synthase
S-(9-deoxy-delta9,12-PGD2)-glutathione
S-(PGJ2)-glutathione
Prostaglandin J2
4-hydroxy-17beta-estradiol-2-S-glutathione
Prostaglandin A2
S-(PGA2)-glutathione

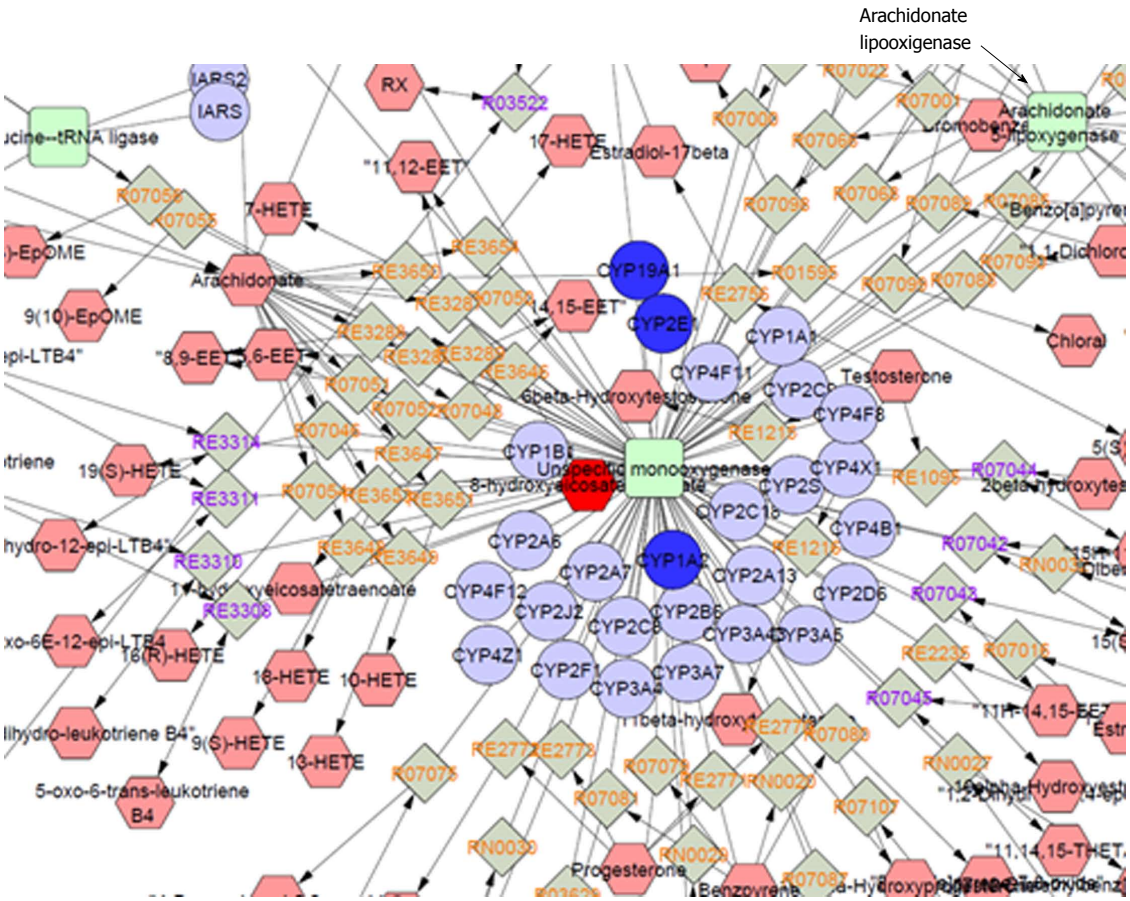



**Figure 3 Whole interactome of compounds (hexagons), chemical reactions (diamonds), enzymes (squares) and genes (circles) associated with nonalcoholic fatty liver disease.** Details on the set of genes and metabolites that were included in the analysis can be found in the main text; terms were filtered according to the ones already found in the databases. The interactome was built using Metscape[73], a plug-in for the widely used network analysis software Cytoscape[74] that supports calculation, analysis and visualization of gene-to-metabolite networks in the context of metabolism.

which encodes for the mitochondrial dimethylglycine dehydrogenase[60]. Betaine (N,N,N-trimethylglicine) performs a critical function in the pathway of methylogenesis by controlling the serum methionine levels; thus, the results of the aforementioned study[61] might be used to tailor therapeutic interventions based on metabolites that modulate the liver methylome.

## INTEGRATION OF DATA DERIVED FROM GENOMICS/PROTEOMICS/ TRANSCRIPTOMICS AND METABOLOMICS TO PREDICT BIOMARKERS ASSOCIATED WITH NAFLD AND NASH

Integration of analyses carried out across multiple biological measurements or OMIC-platforms represents an emerging approach aimed at addressing the challenges imposed by the complex biochemical regulation processes[62].

For example, application of Systems Biology approaches, *i.e.* Gene Set Enrichment Analysis (GSEA)[63], to the field of genomic data has rendered novel knowledge of shared disease-pathways between alcoholic and nonalcoholic liver disease[64]. Likewise, integration of genomic data has highlighted the shared genetic basis of metabolic syndrome and NAFLD[5].

A similar approach can be employed in the field of metabolomics to analyze the enrichment of metabolites that are overrepresented (ORA) in a query-sample against the whole set of metabolites in metabolic pathways. In this context, metabolite set enrichment analysis (MSEA) is the metabolomic counterpart of gene set enrichment. Such analysis, which can be performed by using either commercial or freely available software[65], has been applied to demonstrate alterations in metabolic pathways associated with NAFLD[66].

As a proof of principle, as a part of this work, we performed OMICs-integrative analysis using the IMPaLA (Integrated Molecular Pathway Level Analysis, http://impala.molgen.mpg.de)[67] platform. Briefly, the analysis was conducted by integrating the information on metabolites, genes and proteins, allowing the joint adjusted P-value (Q-value) to be calculated.

Specifically, we selected a list of genes previously associated with NAFLD[5,64], and metabolites that are known to be altered in NAFLD/NASH[68]. Names on metabolites were curated using the compound ID conversion of the web-based MetaboAnalyst tool

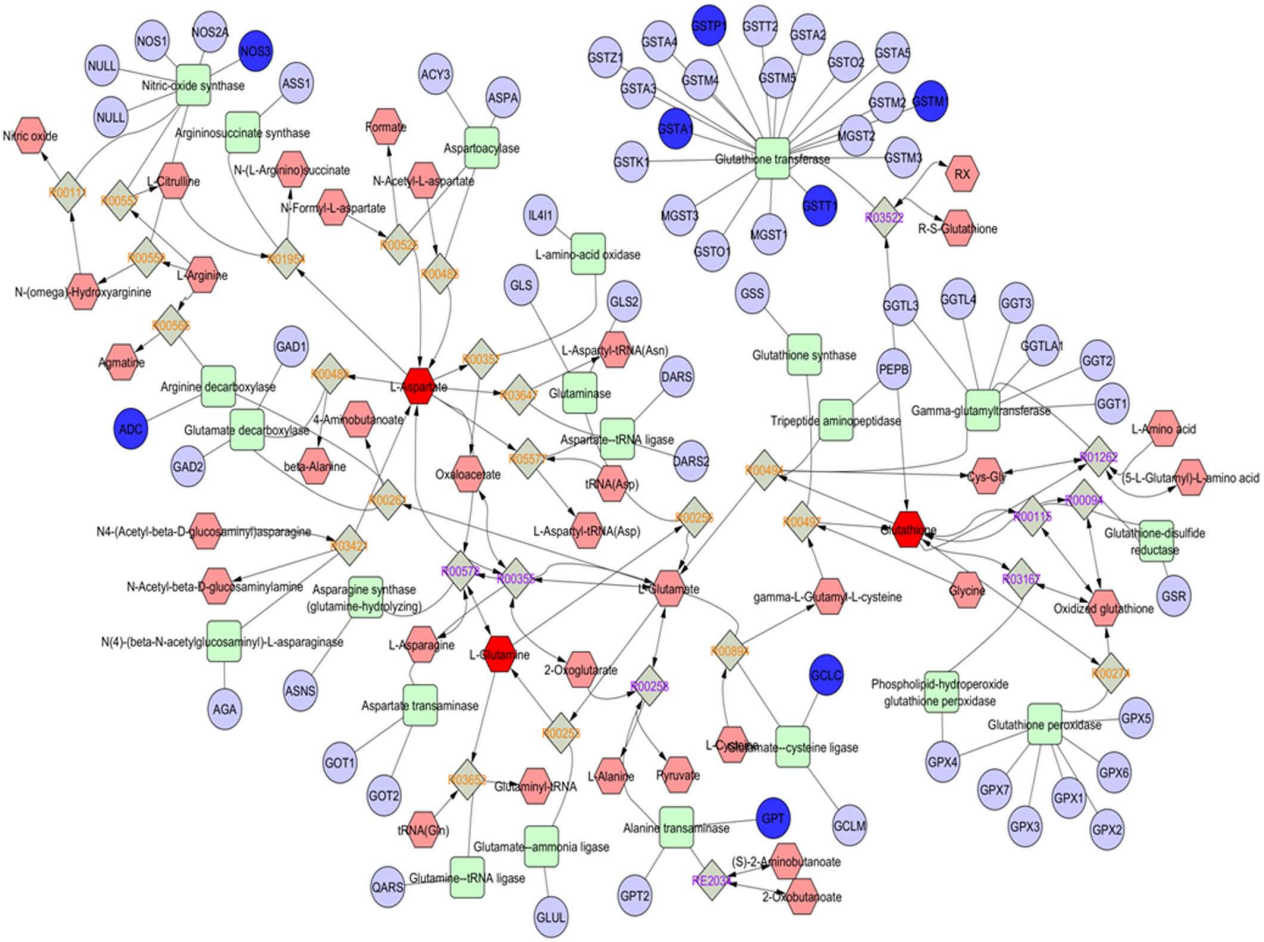



**Figure 4 The urea-cycle, glutamate, and branched-chain amino acids in the biology of nonalcoholic fatty liver disease.** Sub-network analysis showing the urea-cycle and metabolism of amino acids (L-arginine, L-proline, L-glutamate, L-aspartate and L-asparagine) that were extracted from the interactome shown in Figure 3. Compounds (common names in the Human Metabolome Database, http://www.hmdb.ca), chemical reactions, enzymes (KEGG database) and genes (HUGO symbols) are represented by hexagons, diamonds, squares and circles, respectively.

(http://www.metaboanalyst.ca/)[69,70]. We found 2,827 pathways; however, only 219 of 347 input gene-identifiers were mapped to 219 distinct physical entities found in these pathways (with a gene background size of 12655). Similarly, only 32 of 51 input metabolite-identifiers were mapped to 32 distinct physical entities found in the pathways (with a metabolite background size of 5340). Relevant findings, excluding data that was exclusively and heavily dependent on genes or metabolites, are shown in Table 1; pathways and the Q-values for gene and/or metabolite enrichment were jointly calculated.

It is interesting to highlight and discuss a few examples in more detail. For instance, in the pathway "SLC-mediated transmembrane transport" (Reactome database), the overlapping genes and metabolites are *CALM1* (Calmodulin 1), *G6PC* (Glucose-6-Phosphatase Catalytic Subunit), *FGF21* (Fibroblast Growth Factor 21), *GCK* (Glucokinase) and *GCKR* (Glucokinase Regulator), and taurocholic acid, D-mannose, creatinine, L-lactic acid, L-valine, L-isoleucine, L-phenylalanine, L-aspartic acid, L-tyrosine, carnitine, betaine, L-glutamine, linoleic acid, oleic acid, L-leucine and glycocholic acid, respectively.

Another interesting example is the pathway "Transmembrane transport of small molecules" (Reactome database), in which the overlapping genes and metabolites are *G6PC*, *CALM1*, *ATP1A1* (ATPase Na+/K+ Transporting Subunit Alpha 1), *TF* (Transferrin), *ABCC1* (ATP Binding Cassette Subfamily C Member 1), *FGF21*, *GCK*, *GCKR*, *HMOX1* (Heme Oxygenase 1), *ABCB1* (ATP Binding Cassette Subfamily B Member 1), *ABCC2* (ATP Binding Cassette Subfamily C Member 2), *ABCC3* (ATP Binding Cassette Subfamily C Member 3) and *ABCG2* ATP Binding Cassette Subfamily G Member 2), and L-glutamine, D-mannose, creatinine, L-lactic acid, L-valine, L-isoleucine, L-phenylalanine, taurocholic acid, L-aspartic acid, L-tyrosine, carnitine, betaine, linoleic acid, oleic acid, L-leucine and glycocholic acid, respectively.

Finally, in the pathway "Central carbon metabolism in cancer -Homo sapiens (human)" (KEGG database), the overlapping genes and metabolites are *PTEN* (Phosphatase and Tensin Homolog), *EGFR* (Epidermal Growth Factor Receptor), *MET* (MET Proto-Oncogene, Receptor Tyrosine Kinase), *PIK3CA* (Phosphatidylinositol-

**Table 2 List of pathways involved in nonalcoholic fatty liver disease selected from significant *Q*-values independently on whether they represent the effect of gene/s or metabolite/s only**

| Pathway name | Pathway source | Q-joint |
|---|---|---|
| Adipogenesis | Wikipathways | 2.00E-17 |
| Non-alcoholic fatty liver disease (NAFLD) - homo sapiens (human) | KEGG | 2.33E-17 |
| Metabolism | Reactome | 3.72E-17 |
| AGE-RAGE pathway | Wikipathways | 4.22E-17 |
| Vitamin B12 Metabolism | Wikipathways | 5.24E-17 |
| Hepatitis B - homo sapiens (human) | KEGG | 1.79E-16 |
| Folate metabolism | Wikipathways | 1.29E-15 |
| Selenium micronutrient network | Wikipathways | 3.87E-15 |
| TNF signaling pathway - homo sapiens (human) | KEGG | 5.77E-15 |
| JAK-STAT-core | Signalink | 1.99E-14 |
| Adipocytokine signaling pathway - homo sapiens (human) | KEGG | 7.07E-14 |
| Nuclear receptors meta-pathway | Wikipathways | 1.26E-13 |
| IL1 and megakaryocytes in obesity | Wikipathways | 2.73E-13 |
| AGE-RAGE signaling pathway in diabetic complications - homo sapiens (human) | KEGG | 3.79E-13 |
| Spinal cord injury | Wikipathways | 5.44E-13 |
| Malaria - homo sapiens (human) | KEGG | 7.09E-13 |
| Metabolism of lipids and lipoproteins | Reactome | 7.09E-13 |
| SLC-mediated transmembrane transport | Reactome | 1.23E-12 |
| Pathways in cancer - homo sapiens (human) | KEGG | 1.41E-12 |
| Inflammatory bowel disease (IBD) - homo sapiens (human) | KEGG | 2.25E-12 |
| Lung fibrosis | Wikipathways | 2.63E-12 |
| Integrated pancreatic cancer pathway | Wikipathways | 3.10E-12 |
| PI3K-Akt signaling pathway - homo sapiens (human) | KEGG | 3.28E-12 |
| Chagas disease (American trypanosomiasis) - homo sapiens (human) | KEGG | 4.67E-12 |
| HIF-1 signaling pathway - homo sapiens (human) | KEGG | 4.67E-12 |
| AMPK signaling pathway - homo sapiens (human) | KEGG | 9.56E-12 |
| Transmembrane transport of small molecules | Reactome | 9.66E-12 |
| Central carbon metabolism in cancer - homo sapiens (human) | KEGG | 1.41E-11 |
| Jak-STAT signaling pathway - homo sapiens (human) | KEGG | 5.75E-11 |
| DNA damage response (only ATM dependent) | Wikipathways | 7.27E-11 |
| Cytokine-cytokine receptor interaction - homo sapiens (human) | KEGG | 1.01E-10 |
| Longevity regulating pathway - homo sapiens (human) | KEGG | 1.02E-10 |
| Toll-like receptor signaling pathway | Wikipathways | 2.12E-10 |
| Toll-like receptor signaling pathway - homo sapiens (human) | KEGG | 3.94E-10 |
| Toxoplasmosis - homo sapiens (human) | KEGG | 4.73E-10 |
| ABC transporters - homo sapiens (human) | KEGG | 5.94E-10 |
| Transport of glucose and other sugars bile salts and organic acids metal ions and amine compounds | Wikipathways | 8.40E-10 |
| Leukotriene biosynthesis | HumanCyc | 8.71E-10 |
| Insulin resistance - homo sapiens (human) | KEGG | 1.14E-09 |
| Transport of glucose and other sugars bile salts and organic acids metal ions and amine compounds | Reactome | 1.91E-09 |
| Sudden infant death syndrome (SIDS) susceptibility pathways | Wikipathways | 2.12E-09 |
| Cytokines and inflammatory response | Wikipathways | 2.17E-09 |
| AP-1 transcription factor network | PID | 2.22E-09 |
| FoxO signaling pathway - homo sapiens (human) | KEGG | 3.05E-09 |
| Leptin signaling pathway | Wikipathways | 3.57E-09 |
| Transport of inorganic cations-anions and amino acids-oligopeptides | Wikipathways | 4.27E-09 |
| Oncostatin M signaling pathway | Wikipathways | 5.72E-09 |
| Focal adhesion-PI3K-Akt-mTOR-signaling pathway | Wikipathways | 6.53E-09 |
| Amino acid and oligopeptide SLC transporters | Reactome | 1.10E-08 |
| Apoptosis | Wikipathways | 1.41E-08 |
| Apoptotic signaling pathway | Wikipathways | 1.41E-08 |
| Photodynamic therapy-induced NF-kB survival signaling | Wikipathways | 1.84E-08 |
| JAK STAT molecularvariation 1 | INOH | 2.04E-08 |
| MAPK signaling pathway | Wikipathways | 2.04E-08 |
| Aryl hydrocarbon receptor | Wikipathways | 2.35E-08 |
| Transport of inorganic cations/anions and amino acids/oligopeptides | Reactome | 2.40E-08 |
| tRNA aminoacylation | Wikipathways | 3.03E-08 |
| gamma-glutamyl cycle | HumanCyc | 3.61E-08 |
| Glucose homeostasis | Wikipathways | 4.08E-08 |
| Validated transcriptional targets of AP1 family members Fra1 and Fra2 | PID | 4.13E-08 |
| Hepatitis C and hepatocellular carcinoma | Wikipathways | 4.26E-08 |
| Calcineurin-regulated NFAT-dependent transcription in lymphocytes | PID | 4.29E-08 |
| Prostate cancer - homo sapiens (human) | KEGG | 4.29E-08 |
| Tuberculosis - homo sapiens (human) | KEGG | 4.45E-08 |
| Apoptosis - homo sapiens (human) | KEGG | 4.54E-08 |

| | | |
|---|---|---|
| tRNA charging | HumanCyc | 5.96E-08 |
| Transcription factor regulation in adipogenesis | Wikipathways | 6.27E-08 |
| Sterol regulatory element-binding proteins (SREBP) signalling | Wikipathways | 6.27E-08 |
| Integrated lung cancer pathway | Wikipathways | 6.43E-08 |
| TNF related weak inducer of apoptosis (TWEAK) signaling pathway | Wikipathways | 8.14E-08 |
| mRNA protein and metabolite induction pathway by cyclosporin A | Wikipathways | 8.47E-08 |
| PPAR signaling pathway | Wikipathways | 9.54E-08 |
| Immune system | Reactome | 9.57E-08 |
| Regulation of lipid metabolism by peroxisome proliferator-activated receptor alpha (PPARalpha) | Wikipathways | 1.13E-07 |
| AMP-activated protein kinase (AMPK) signaling | Wikipathways | 1.34E-07 |
| Photodynamic therapy-induced NFE2L2 (NRF2) survival signaling | Wikipathways | 1.52E-07 |
| Leptin insulin overlap | Wikipathways | 1.65E-07 |
| Class I MHC mediated antigen processing and presentation | Wikipathways | 1.73E-07 |
| Caspase cascade in apoptosis | PID | 1.99E-07 |
| Overview of nanoparticle effects | Wikipathways | 2.17E-07 |
| Alpha6Beta4Integrin | NetPath | 2.29E-07 |
| VEGFA-VEGFR2 signaling pathway | Wikipathways | 2.30E-07 |
| HIV-1 Nef: Negative effector of Fas and TNF-alpha | PID | 2.65E-07 |
| Innate immune system | Reactome | 2.69E-07 |
| $Na^+/Cl^-$ dependent neurotransmitter transporters | Reactome | 3.10E-07 |
| Colorectal cancer - homo sapiens (human) | KEGG | 3.42E-07 |
| Regulation of toll-like receptor signaling pathway | Wikipathways | 3.64E-07 |
| stress induction of hsp regulation | BioCarta | 3.64E-07 |
| Amino acid transport across the plasma membrane | Reactome | 3.72E-07 |
| Programmed cell death | Reactome | 3.85E-07 |
| Apoptosis modulation and signaling | Wikipathways | 4.42E-07 |
| SREBF and miR33 in cholesterol and lipid homeostasis | Wikipathways | 4.84E-07 |
| JAK STAT pathway and regulation | INOH | 5.42E-07 |

OMICs-integrative analysis was performed using the IMPaLA (Integrated Molecular Pathway Level Analysis, http://impala.molgen.mpg.de)[67] platform. A joined adjusted *P*-value (*Q*-value) was calculated to control for multiple testing by false discovery rate.

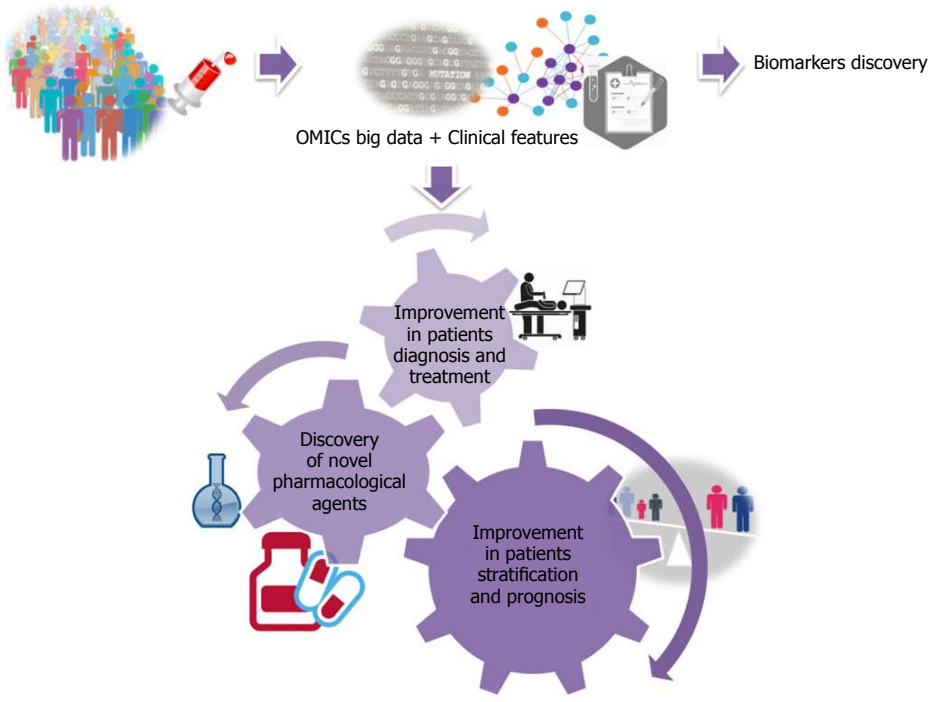



**Figure 5 What to expect for the near future.** A personalized nonalcoholic fatty liver disease approach by integrating OMICs big data with clinical information.

4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha), *MTOR* (Mechanistic Target Of Rapamycin Kinase), *AKT2* (AKT Serine/Threonine Kinase 2) and *GCK*, and L-glutamine, L-lactic acid, L-valine, L-isoleucine, L-phenylalanine, L-aspartic acid, L-tyrosine and L-leucine, respectively.

From these few examples, we may conclude that some pathways such as solute carrier (SLC) transporters should be further explored; in fact, available experimental data, while limited, support the participation of ABCC-family in NAFLD pathophysiology[71].

Nonetheless, the findings discussed above do not

necessarily indicate that no other important pathways are potentially involved in the biology of NAFLD. In fact, Table 2 illustrates the myriad of processes involved in the pathogenesis of a complex disease such as NAFLD. In addition, Figure 3 depicts the complexity of the interactome among the whole set of genes, enzymes, chemical reactions and metabolites associated with NAFLD. Figure 4 shows a sub-network emphasizing the importance of the urea-cycle and metabolism of L-arginine, L-proline, L-glutamate, L-aspartate and L-asparagine. Specifically, features in Figure 4 highlight the central role played by aminotransferases and gamma-glutamyl transferases in the frame of altered L-glutamine/L-glutamate, glutathione and BCAA levels, as already mentioned.

Finally, additional biomarkers that target immunity-related pathways, for example circulating levels of cytokines/chemokines, antibodies *etc*. might be useful in predicting NASH progression toward advanced phases[72].

## CONCLUSION

Implementation of OMICs-derived biomarkers in the management and treatment of patients with NAFLD is still under extensive evaluation. Knowledge gained on genetic signatures associated with NAFLD and NASH, as well as the role of circulating cfmiRNAs and plasma metabolites, should be promptly translated into the clinical setting. Nevertheless, rigorous steps that must include validation and replication are mandatory before OMICs biomarkers are ready for use as diagnostic markers to identify patients at risk of advanced disease, including liver cancer.

What to expect for the near future: A personalized NAFLD approach by integration of OMICs - big data and clinical information (Figure 5): (1) It is expected that, in the near future, NAFLD patients can be diagnosed and treated according to their own "molecular signature"; (2) Specific focus should be placed on prevention and early diagnosis by the application of biomarkers of disease risk; (3) Selection of "personalized drugs" as well as tailored therapy should be made according to the specific molecular signature; and (4) Personalized lifestyle intervention is desirable but it is envisioned that the basic and general recommendations about alcohol restriction, healthy diet and exercise would remain the foundation of prevention and therapy.

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**P- Reviewer**: Arslan N, Enomoto H, Jarcuska P, Sutti S, Toshikuni N
**S- Editor**: Ma YJ **L- Editor**: A **E- Editor**: Huang Y

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

*World J Gastroenterol* 2018 April 21; 24(15): 1616-1621

DOI: 10.3748/wjg.v24.i15.1616

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

MINIREVIEWS

# Autonomic nervous system network and liver regeneration

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Author contributions: Kamimura K wrote the manuscript; Inoue R, Nagoya T, Sakai N, Goto R, Ko M, Niwa Y, and Terai S collected information and performed experiments; all authors read and approved the final version of the manuscript.

Supported by the Brain Research Institute Grant, Niigata University.

Institutional review board statement: The study was reviewed and approved by the Institutional Review Board of Niigata University. All animal experiments were approved by and conducted in full compliance with the regulations of the Institutional Animal Care and Use Committee at Niigata University, Niigata, Japan.

Conflict-of-interest statement: The authors declare that they have no current financial arrangement or affiliation with any organization that may have a direct influence on their work.



Manuscript source: Invited manuscript

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Received: March 13, 2018
Peer-review started: March 14, 2018
First decision: March 30, 2018
Revised: April 1, 2018
Accepted: April 16, 2018
Article in press: April 16, 2018
Published online: April 21, 2018

## Abstract

To date, various signal transducers, cytokines, growth factors, and hormones have been reported to play an important role in homeostasis of various organs. Various cells and organs are involved in the hepatic regeneration process, which proceeds as a result of the coordination of many factors. While these factors are well known to be involved in the liver regeneration after the liver injury, however, as the details of such mechanisms have not been sufficiently elucidated, the practical applicability of hepatic regeneration based on the action of these and cytokines growth factors is still unclear. In terms of the involvement of the autonomic nervous system in hepatic regeneration, cell proliferation resulting from direct signal transduction to the liver has also been reported and recent studies focusing on the inter-organ communication *via* neural network opened a novel aspect of this field for therapeutic applicability. Therefore, the appropriate understanding of the relationship between autonomic neural network and liver regeneration through various organs including brain, afferent nerve, efferent nerve, *etc*. is essential. This mini-review explains the principle of neural system involved in the inter-organ communication and its contribution on the liver regeneration upon the liver

injury reviewing recent progress in this field.

**Key words:** Autonomic nerve; Neural network; Liver regeneration; Hormone



**Core tip:** The review of the relationship between autonomic neural network and liver regeneration shows that an inter-organ communication is functioning in a coordinated manner through the autonomic nervous system as a biological mechanism for hepatic regeneration and functional maintenance when the liver is damaged. Therefore, this mini-review presents how autonomic nerve fibers affect hepatic regeneration including the results of our most recent research.

Kamimura K, Inoue R, Nagoya T, Sakai N, Goto R, Ko M, Niwa Y, Terai S. Autonomic nervous system network and liver regeneration. *World J Gastroenterol* 2018; 24(15): 1616-1621 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i15/1616.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i15.1616

## INTRODUCTION

Many studies on the mechanisms of hepatic regeneration have led to the identification of cytokines, growth factors, and signal transducers that are produced by Kupffer cells and other cell types[1-17] which may play an important role in homeostasis of the liver (Table 1). These cytokines and growth factors are thought to cause cells enlargement and proliferation, resulting in functional recovery. However, as the details of such mechanisms have not been sufficiently elucidated, the practical applicability of hepatic regeneration based on the action of cytokines and growth factors is still unclear. Various cells and organs are involved in the hepatic regeneration process, which proceeds as a result of the coordination of many factors.

Moreover, in terms of the involvement of the autonomic nervous system in hepatic regeneration, cell proliferation resulting from direct signal transduction to the liver has also been reported[18-20].

More specifically, this refers to actions accompanying the activation of the parasympathetic nervous system, which mediates the hepatic branch of the vagus nerve. The autonomic nervous system was first described by John N Langley in 1916[21] as an important mechanism that maintains homeostasis in organisms. These nerves are distributed internally within the blood vessels, heart, lungs, gastrointestinal tract, liver[22,23], and reproductive organs and are controlled by a feedback system that is mainly situated in the brain. While the functions of the sympathetic and parasympathetic nerves comprising the autonomic nervous system have been considered as antagonistic, they are now believed to play an important organ-related role as part of a network that maintains homeostasis in organisms. This inter-organ communication is very important in various pathologies. For example, intestinal bacterial flora have been found to be strongly involved in Parkinson's disease[24] and autism[25], and a system to control blood sugar has been discovered in which pancreatic beta cells proliferate *via* autonomic nervous system signaling following a hepatectomy[26,27-29].

These results suggest that an inter-organ network functions in a coordinated manner through the autonomic nervous system as a biological mechanism for hepatic regeneration and functional maintenance when the liver is damaged[30]. This mini-review describes how autonomic nerve fibers affect hepatic regeneration based on our recent research results.

**Table 1 Factors related to the liver regeneration**

| Factors related to liver regeneration | Ref. |
|---|---|
| Tumour necrosis factor | [3] |
| Interleukin 6 | [3] |
| NF-kappa B | [4] |
| Oncostatin M | [5] |
| Signal transducers and activator of transcription 3 | [6] |
| Hepatocyte growth factor | [7-9,11,16] |
| Epidermal growth factor | [10] |
| Transforming growth factor alpha | [10,14] |
| Epidermal growth factor receptor | [12,13] |
| Platelets | [15,16] |
| Insulin-like growth factor 1 | [16] |
| Lymphotoxin beta receptor | [17] |
| Insulin | [38] |
| Glucagon | [38] |
| Epidermal growth factor | [38] |
| Serotonin | [41,43,45,47] |
| Serotonin S2 receptor | [33] |
| Beta-catenin | [2] |
| Autonomic nervous system (direct feedback) | [18,20,31,32] |

## IMPACT OF AUTONOMIC NERVE FIBERS DISTRIBUTED IN THE LIVER ON HEPATIC REGENERATION

Reportedly, in addition to humoral factors, the autonomic nervous system is also involved in the hepatic regeneration process. Most studies have examined direct feedback relationship between the liver and brain. Signals starting in the liver are transmitted *via* the afferent sympathetic nervous system to the ventromedial region of the hypothalamus and then to the lateral region of the hypothalamus; they then pass through the dorsal nucleus of the vagus nerve of the medulla oblongata after which they return to the liver[18,19]. Kiba *et al*[20]. reported that in rats that had undergone partial hepatectomy, hepatic regeneration was slow when the hepatic branch of the vagus nerve

was resected. In addition, they demonstrated that when the ventromedial region of the hypothalamus, which is the center of the sympathetic nervous system, was destroyed, vagus nerve signal transduction became excessive, thereby promoting post-hepatectomy hepatic regeneration[31,32]. This phenomenon occurred even if hepatectomy was not performed, and chronological evaluation showed that cell proliferation in the hepatic regeneration process peaked on day 3[33].

Thus, autonomic nervous system feedback throughout the liver is involved in hepatocyte proliferation. Kiba *et al*[34] also demonstrated that when the ventromedial hypothalamus, the center of the efferent sympathetic nervous system, was destroyed, pancreatic beta cells and extrapancreatic secretory cells proliferated, activating the growth of epithelial cells in the gastrointestinal tract[35]. These results demonstrated the importance of the efferent vagus nerve in the activation of cell proliferation in various organs and suggested that when the liver is injured, neural signals relayed from the liver to various organs through the brain and efferent vagus nerve might contribute to homeostasis maintenance in the body.

## IMPACT OF INTER-ORGAN NETWORKS MEDIATING HEPATIC REGENERATION *VIA* THE AUTONOMIC NERVOUS SYSTEM

Based on the suspected contribution of the neural network on hepatic regeneration after liver injury, we focused on the effect of various organs on liver regeneration after liver injury with respect to this network. While no reports on liver regeneration have focused on this neural network, several studies so far have focused on the pancreas as an organ controlled by the autonomic nervous system[26,27-29,36]. Briefly, marked proliferation of beta cells was noted in the pancreas following partial hepatectomy in response to signal transduction through the afferent sympathetic nervous system, brain, and efferent vagus nerve[27]. Additionally, similar results were obtained when gene transfer promoted the afferent signal transduction by ERK activation[26]. These results provide evidence that the autonomic nervous system is important in maintaining for blood sugar homeostasis after severe liver damage.

It appears that this autonomic nervous system first activates the afferent sympathetic nerve in the damaged liver, which transduces the signal to the center of the autonomic nervous system in the brain and then to the efferent vagus nerve; this results in the activation of cell proliferation in various organs inside the abdominal cavity, such as the liver, gastrointestinal tract organs, and pancreas. However, no study has focused on the effect of this system on liver regeneration. Therefore, here, we focus on this system as an effector of liver regeneration. While various factors might be involved, GI hormones are known to play an important role in hepatic regeneration.

Fujita *et al*[37] administered various gastrointestinal and pancreatic hormones, including glucagon, secretin, and cholecystokinin, to mice that had undergone partial hepatectomy and investigated their effects on hepatic regeneration based on weight changes and histological findings. They found that the mice exhibited liver weight increases of approximately 50% following glucagon and insulin administration and suggested that this effect was mainly due to hypertrophy of the remaining cells. Lai *et al*[38] also reported that the combined administration of glucagon and insulin was effective for hepatic regeneration in rats that had undergone partial hepatectomy. These results demonstrated that gastrointestinal hormones may play an important role in hepatic regeneration.

Many gastrointestinal hormones assist in the maintenance of homeostasis in living organisms. Among these, serotonin, which is emitted by chromaffin cells in the intestines, has been known to encourage proliferation of liver cells[39,40]. Serotonin is a monoamine neurotransmitter that is synthesized by the enzyme tryptophan hydroxylase 1 in chromaffin cells[41] and is released in the intestine[26,27] when the parasympathetic nervous system is activated[42]. Platelet granules also contain serotonin, which is released when platelets come into contact with liver cells, after which it functions as a growth factor for liver cells[40,41,43] through the 5-$HT_2$ receptors. Moreover, it has been reported that mice deficient in tryptophan hydroxylase 1 exhibit poor liver regeneration after hepatectomy[41].

In addition, Matondo *et al*[44] reported that although serotonin transporter depletion disturbed biological homeostasis, a small amount of serotonin in the liver was sufficient for liver regeneration. Mechanistically, DNA synthesis in primary rat hepatocytes cultures was induced by serotonin[33], but it was arrested by 5-HT2 receptor blockade at $G_1$/S transition[15,45]. In the pathophysiological state, reduction of serotonin reuptake transporter function caused insulin resistance and hepatic steatosis independent of the food intake[46]; serotonin protected mouse liver from cholestatic injury by stabilizing the bile salt pool after bile duct ligation through adaptation of renal transporters in cholestasis[47].

Thus, while serotonin has been reported to act as a growth factor, no analyses of an intra-organ network, focusing on increased serotonin production in the small intestine through the afferent sympathetic nervous system and the efferent vagus nerve branch, or the promotion of hepatic regeneration have been reported.

These reports provide evidence that gastrointestinal hormones may function as growth factors upon hepatic injury. Therefore, recent studies are focusing on inter-organ communication between the liver and GI tract upon liver injury. We have reported that the effect of serotonin on liver regeneration following liver injury is mediated through this neural relay, which

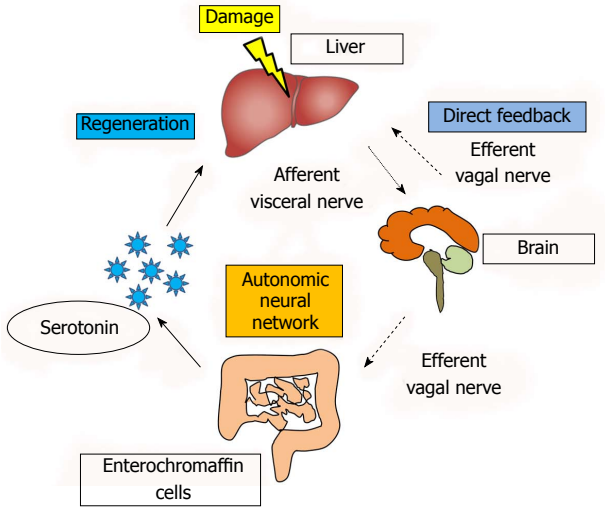


**Figure 1 Involvement of neural signals in liver regeneration.** This figure is partly reused and modified with updated information from Figure 1 in reference 49 with their permission.

begins in the liver and passes through the brain and GI tract and then returns to the liver[48,49]. Our results demonstrated that the partial hepatectomy increases the serotonin release from the GI tract contributing to the liver regeneration which were evidenced by the proliferating cell nuclear antigen, BrdU incorporation and liver weight-to-body weight ratio[48,49] (see details in reference 48). In addition, this activation was blocked by neuronal blockade of the afferent visceral nerve by capsaicin suggesting that the activation of the afferent visceral nerve from the liver to the efferent vagal nerve through the brain is important[48]. Therefore, the neural relay significantly contributes in the liver regeneration upon the liver injury by activating the release of GI tract hormones as a part of maintenance of homeostasis. A summary of our studies and the reported effects of the autonomic nervous system are shown in Figure 1. This figure illustrate that while the traditionally studies have shown the direct feedback between the liver and brain have been reported, neural relay signal communicating various organs is also important, which starts from the damaged liver to the GI tract through the afferent visceral nervous system, brain, and the efferent vagal nervous system contributes to activate and release the serotonin from enterochromaffin cells to promote liver regeneration. As various factors are related to the liver regeneration (Table 1), it is obvious however, further studies are necessary to clarify the relationship between the factors.

## CONCLUSION

The review of the relationship between autonomic neural network and liver regeneration shows that an inter-organ network is functioning in a coordinated manner through the autonomic nervous system as a biological mechanism for hepatic regeneration and functional maintenance when the liver is damaged. We believe that this study may represent a step toward the development of essential therapeutics to promote liver regeneration.

## ACKNOWLEDGMENTS

The authors would like to thank Takao Tsuchida, Division of Gastroenterology and Hepatology, Niigata University for his excellent assistance in preparing the figure.

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**P- Reviewer**: Boscá L, Ilangumaran S, Nagaya M **S- Editor**: Wang XJ
**L- Editor**: A **E- Editor**: Huang Y

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

*World J Gastroenterol* 2018 April 21; 24(15): 1622-1631

DOI: 10.3748/wjg.v24.i15.1622

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Basic Study

# Evaluation of safety for hepatectomy in a novel mouse model with nonalcoholic-steatohepatitis

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Author contributions: Ozawa Y and Tamura T designed the study and wrote the initial draft of the manuscript; Ozawa Y, Owada Y, Shimizu Y and Kemmochi A performed the animal experiments, the biochemical analysis and gene expression analysis; Ozawa Y, Tamura T, Matsuzaka T and Shimano H contributed to the analysis and interpretation of the data; all of the other authors have critically reviewed the manuscript; the final version of the manuscript was approved by all of the authors.

Supported by Ministry of Education, Culture, Sports, Science, and Technology of Japan, KAKENHI, No. 26861059 and No. 16K10489.

Institutional review board statement: Animal experiments were performed in accordance with the university's Regulations for Animal Experiments and Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions, under the jurisdiction of the Japanese Ministry of Education, Culture, Sports, Science, and Technology.

Institutional animal care and use committee statement: Animal experiments were performed in human manner after receiving approval from Institutional University Experiment Committee of University of Tsukuba (protocol number: 17-312).

Conflict-of-interest statement: The authors report no relevant conflicts of interest.

Data sharing statement: No data are available.

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



Manuscript source: Unsolicited manuscript

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Received: February 14, 2018
Peer-review started: February 14, 2018
First decision: March 9, 2018
Revised: March 16, 2018
Accepted: March 25,2018
Article in press: March 25,2018
Published online: April 21, 2018

## Abstract

***AIM***

To investigate whether the liver resection volume in a newly developed nonalcoholic steatohepatitis (NASH) model influences surgical outcome.

***METHODS***

For establishment of a NASH model, mice were fed a high-fat diet for 4 wk, administered $CCl_4$ for the last 2 wk, and administered T0901317 for the last 5 d. We divided these mice into two groups: A 30% partial hepatectomy (PH) of NASH liver group and a 70% PH of NASH liver group. In addition, a 70% PH of normal liver group served as the control. Each group was evaluated for survival rate, regeneration, apoptosis, necrosis and DNA expression after PH.

***RESULTS***

In the 70% PH of NASH group, the survival rate was significantly decreased compared with that in the control and 30% PH of NASH groups ($P < 0.01$). 10 of 32 mice in the NASH 70% PH group died within 48 h after PH. Serum aspartate aminotransferase (AST) levels and total bilirubin (T-Bil) in the NASH 70% PH group were significantly higher than the levels in the other two groups (AST: $P < 0.05$, T-Bil: $P < 0.01$). In both PH of NASH groups, signaling proteins involved in regeneration were expressed at lower levels than those in the control group ($P < 0.01$). The 70% PH of NASH group also exhibited a lower number of Ki-67-positive cells and higher rates of apoptosis and necrosis than the NASH 30% PH group ($P < 0.01$). In addition, DNA microarray assays showed differences in gene expression associated with cell cycle arrest and apoptosis.

***CONCLUSION***

The function of the residual liver is impaired in fatty liver compared to normal liver. A larger residual volume is required to maintain liver functions in mice with NASH.


**Key words:** Hepatectomy; Liver regeneration; Residual liver; Liver proliferation; Nonalcoholic steatohepatitis



**Core tip:** We report whether the liver resection volume in the nonalcoholic steatohepatitis (NASH) model influences surgical outcome. The population of patients with NASH has been increasing. However, few animal models fully reflect both the histopathology and pathophysiology of NASH in humans. We established a novel experimental NASH model that exhibited the same characteristics as NASH in humans. This study elucidates the metabolism of the residual liver after a hepatectomy with NASH. Compared with normal liver, the residual NASH liver function is impaired, especially its regenerative ability. Therefore, a larger residual volume is required to maintain liver function in NASH liver after partial hepatectomy.

Ozawa Y, Tamura T, Owada Y, Shimizu Y, Kemmochi A, Hisakura K, Matsuzaka T, Shimano H, Isoda H, Ohkohchi N. Evaluation of safety for hepatectomy in a novel mouse model with nonalcoholic-steatohepatitis. *World J Gastroenterol* 2018; 24(15): 1622-1631 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i15/1622.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i15.1622

## INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is observed in 20%-40% of the general population, and its incidence continues to increase in industrialized countries[1,2]. NAFLD includes several diseases, such as simple liver steatosis, nonalcoholic steatohepatitis (NASH), and cirrhosis. NASH is characterized by hepatic steatosis, lobular inflammation, and abnormal glucose tolerance. In NASH, continuous inflammation contributes to hepatocellular carcinoma (HCC)[3,4]. The cause of HCC is frequently infection with hepatitis B virus and hepatitis C virus (HCV). New antiviral medications for hepatitis are currently being used in clinics; therefore, the number of patients with virus-related HCC is expected to decrease in the future[5-9]. By contrast, the number of patients with NASH-related HCC has been increasing recently, and this trend is expected to continue because no effective treatments are available[10].

Steatosis is a risk factor for postoperative liver failure[11,12]. A number of clinical studies revealed that steatosis caused severe mortality and morbidity after liver resection compared with normal liver following liver resection[11,12]. In experimental models, hepatectomy of fatty livers resulted in suppressed liver regeneration and survival rates[13-16]. However, the influence of hepatectomy on NASH livers has not been extensively evaluated.

Hepatectomy is a standard and most effective therapy for HCC patients. Postoperative liver failure is a serious complication after hepatectomy, and its occurrence correlates with the volume and function of the residual liver[17-20]. To prevent liver failure after hepatectomy, the liver resection volume is limited according to preoperative liver function[21-23]. For promotion of regeneration and maintaining liver function preserving sufficient residual liver volume enables the prevention of liver failure[24,25]. Thus, the degree of liver regeneration is dependent on the volume of the residual liver. Although several NASH models, such as the methionine- and choline-deficient (MCD) model and high-fat (HF) diet model, have been reported, few models completely reflect the histopathology and pathophysiology of NASH in humans[26,27]. The disadvantages of the MCD model are that MCD mice exhibit severe body weight loss with the absence of insulin resistance. The HF diet model is not suitable for researching the pathogenesis of NASH because a longer period of time is required for presentation of NASH characteristics, and hepatic fibrosis is weaker than that observed in human NASH.

Thus, the ability of regeneration in the NASH liver has not yet been assessed in an experimental model. For the same reasons, the effect of hepatectomy on NASH liver has not been clearly elucidated in previous reports. We established a novel experimental NASH model that indicates similar histopathological and pathophysiological characteristics as those of NASH in humans[28]. The aim of this study was to investigate whether a difference in liver resection volume in a novel NASH model influences surgical outcomes.

## MATERIALS AND METHODS

### Animals

Six-week-old male C57BL/6J mice were obtained from Charles River Laboratories Japan, Inc. (Kanagawa, Japan) and were acclimated for one week before the start of the experiment. Mice were maintained under a 12-h light-dark cycle and had free access to standard chow and tap water. The animal experiments were performed in a humane manner after receiving approval from the Institutional University Experiment Committee of the University of Tsukuba and in accordance with the Regulations for Animal Experiments at the university and Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science, and Technology.

### NASH mouse model protocol

NASH mice were fed an HF diet (60 kcal% fat; D12492, Research Diets, Inc., New Brunswick, NJ, United States) for 4 wk, intraperitoneally injected with $CCl_4$ (Wako Pure Chemical Industries, Ltd., Osaka, Japan) twice a week for the final 2 wk, and intraperitoneally injected with T0901317 (Cayman Chemical Co., Ann Arbor, MI, United States) solubilized in DMSO for the final 5 d. The $CCl_4$ dose was 0.1 mL/kg, and the T0901317 dose was 2.5 mg/kg[28].

### Surgical procedure and anesthesia

We categorized the mice into three groups: (1) 70% partial hepatectomy (PH) of normal liver mice as the control; (2) 30% PH of NASH liver group; and (3) 70% PH of NASH liver group. The normal liver mice have been not added any reagent and the histology and pathology have been not change. In 30% PH and 70% PH of the NASH liver group, liver specimens were evaluated by an experienced pathologist in a blinded fashion, the histology and pathology finding in the NASH severity of each groups have resulted in no difference in the NAFLD activity scores[28]. All mice received the hepatectomy 48 h after the final administration of $CCl_4$ and T0901317. In the 70% PH groups, the left and middle lobes of the liver were removed by using a single ligature, whereas only the left lobe was removed in the 30% PH group[29]. Hepatectomy was performed under ether anesthesia.

### Liver tissue collection

Blood samples were collected from the orbital capillary and centrifuged at 3000 rpm for 10 min to isolate the serum. Each sample was stored at -80 ℃ until analysis. Mice of each group were sacrificed at 6 h and 12 h after PH. Then, the liver was quickly removed and weighed. The liver specimen was immediately fixed in 10% neutral-buffered formalin for further histological examination. Survival rates were evaluated in the NASH 70% PH group ($n$ = 32) and NASH 30% PH group ($n$ = 27).

### Histology and immunohistochemistry

Fixed liver tissues were processed and embedded in paraffin using standard methods. Then, liver tissues were sliced into 2-μm thick paraffin sections and stained with hematoxylin and eosin (HE) to evaluate necrosis. Necrotic areas were detected by morphological features, and the ratio of necrosis/total area was calculated in 20 random intralobular fields. Liver proliferation was assessed by Ki-67 staining. Apoptosis was detected by TUNEL staining. TUNEL staining and Ki-67 staining were performed using an antibody kit (New History Science Laboratory Co., Ltd., Tokyo, Japan). The ratio of positive/total hepatocytes was calculated in 20 random intralobular fields.

### Immunoblotting

Liver tissue extracts were prepared from specimens that were frozen in liquid nitrogen. We evaluated the expression of signaling proteins involved in liver regeneration, including AKT, STAT3, and ERK1/2, by western blotting. We compared the expression levels of these proteins in each group 6 h after PH. Immunoblots were developed using polyclonal antibodies against phospho-AKT (9271), total AKT (9272), phospho-STAT3 (9131), total STAT3 (9132), phospho-ERK1/2 (9101), and total ERK1/2 (9102) (Cell Signaling Technology, Beverly, MA, United States).

### Gene expression analysis

Liver tissue samples were freshly collected and immediately frozen at -30 ℃ until investigation. Frozen liver samples were homogenized, and total RNA was isolated from whole cells using a NucleoSpin® RNA kit (Takara Bio, Inc., Otsu, Japan). RNA concentrations were determined by measuring the absorbance at 260/280 nm with a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Inc., Wilmington, DE, United States). Synthesis of complementary DNA was performed using AMV Reverse Transcriptase (Promega, Corp., Madison, WI, United States) and random primers (Takara Bio, Inc., Otsu, Japan). Briefly, a mixture of 1 mmol/L dNTPs (Fermentas Life Sciences, Inc., Burlington, ON, Canada), 0.025 μg/mL random primers, 0.25 U/mL reverse transcriptase, and 500 ng of total RNA was incubated at 30 ℃ for 10 min, 37 ℃ for 60 min, 95 ℃ for 5 min and

**Table 1 Serum parameters of normal liver and nonalcoholic steatohepatitis groups after partial hepatectomy**

| | 6 h after PH | | | | 12 h after PH | | | |
|---|---|---|---|---|---|---|---|---|
| | AST | ALT | T-Bil | IL-6 | AST | ALT | T-Bil | IL-6 |
| Normal 70%PH | 2343.3 ± 6160.4 | 1828.3 ± 990.4 | 1.43 ± 0.5 | 2036.0 ± 1470.9 | 2976.7 ± 1395.7 | 2053.3 ± 886.2 | 2.0 ± 0.9 | 731.5 ± 483.7 |
| NASH 30%PH | 1610.0 ± 3700.6 | 1507.1 ± 563.5 | 0.76 ± 0.2 | 1511.5 ± 284.8 | 1841.3 ± 619.1 | 1522.9 ± 537.9 | 0.9 ± 0.7 | 692.8 ± 211.1 |
| NASH 70%PH | 3064.3 ± 1289.8[a] | 2422.9 ± 1194.8 | 2.57 ± 1.36[b] | 3026.2 ± 2127.5 | 4067.5 ± 2059.2[a] | 2403.8 ± 1111.8 | 3.85 ± 0.96[b] | 987.9 ± 550.7 |

Data are presented as the mean ± SD ($n$ = 5-7). [a]$P$ < 0.05, [b]$P$ < 0.01 *vs* other groups. NASH: Nonalcoholic steatohepatitis; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; PH: Partial hepatectomy; T-Bil: Total bilirubin; IL-6: Interleukin-6.

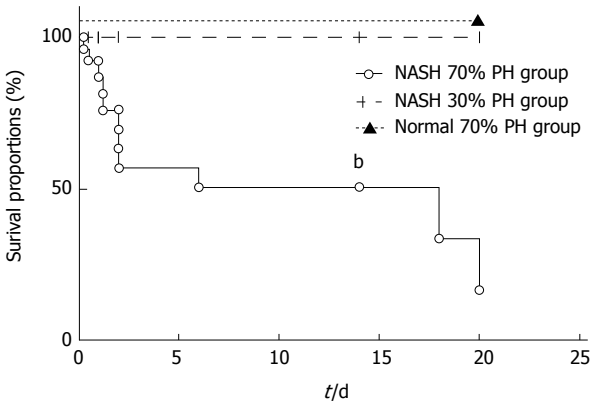


**Figure 1 Survival rate after partial hepatectomy.** Survival rates of the 30% PH and 70% PH groups were evaluated by the Kaplan-Meier method. All mice in the 30% PH group survived. In contrast, 80% of the NASH 70% PH group died by 20 d. $n$ = 27 in NASH 30% PH group; $n$ = 32 in NASH 70% PH group. [b]$P$ < 0.01. PH: partial hepatectomy; NASH: nonalcoholic steatohepatitis.

4 ℃ before storage at -80 ℃.

RT-PCR primers were designed using Primer Express Software for Real-time PCR ver. 3.0 (Applied Biosystems, Inc., Foster City, CA, United States) based on the sequences available in GenBank. Primers were purchased from Takara Bio, Inc. (Otsu, Japan). *GADD45A* primer sequences were 5′-CCTGCACTGTGTGCTGGTGA-3′ and 5′-CCACTGATCCATGTAGCGACTTTC-3′. *PDE4B* primer sequences were 5′-CCCATCAGCAGTTAAGGACAGGA-3′ and 5′-TGGGCAGAACTAGGGACTCAAGA-3′.

Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an endogenous control. RT-PCR was performed using SYBR-Green Real-Time PCR Master Mix-Plus (Toyobo Co., Ltd., Osaka, Japan) and an Applied Biosystems 7300 real-time PCR system (Applied Biosystems, Inc., Foster City, CA, United States) as recommended by the manufacturer's instructions[28].

### Microarray analysis

DNA microarray analysis was conducted on RNA samples isolated from liver tissue in the control group and novel NASH model group. Labeled cRNA was synthesized from 100 ng of total RNA using a GeneChip® 3′ IVT Plus Reagent Kit (Affymetrix, Inc., Santa Clara, CA, United States) according to the manufacturer's protocol. Fragmented and labeled cRNA (7.5 μg) was hybridized to an Affymetrix Mouse MG-430 PM Array Strip (Affymetrix) for 16 h at 45 ℃. The strips were washed and stained using a GeneAtlas Fluidics Station 400 (Affymetrix), and the resulting images were scanned using a GeneAtlas Imaging Station (Affymetrix). Probe-level analysis, including background subtraction and quantile normalization, was conducted using a robust multiarray average algorithm (RMA) using Affymetrix Expression Console Software 1.4 (Affymetrix). The gene expression profile of the novel NASH model was compared with the HF group. Genes exhibiting differences in expression with an increase of greater than 1.4-fold and a decrease of less than 0.65-fold were classified as differentially expressed genes[28].

### Statistical analysis

All data are expressed as the mean ± SD. Statistical analyses were conducted using PRISM. Mann-Whitney *U* test was used for comparing between two groups. *P*-values less than 0.05 were considered significant. The Kaplan-Meier estimator was used for survival rate evaluation.

## RESULTS

### Survival rate

The survival rate of the NASH 70% PH group was significantly lower than that of the NASH 30% PH group ($P$ < 0.01) (Figure 1), and 10 of 32 mice in the NASH 70% PH group died within 48 h after PH. On the other hand, all mice in the NASH 30% PH group survived.

### Liver function

At 6 and 12 h after PH, serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were high in all three groups. AST levels in the NASH 70% PH group were significantly higher than the levels in the other two groups (AST: $P$ < 0.05). Total bilirubin (T-Bil) in the normal liver and NASH 30% PH groups did not change, but the values only significantly increased in the NASH 70% PH group ($P$ < 0.01) (Table 1).

### Liver proliferation assay

Many more Ki-67-positive hepatocytes were observed in the NASH 30% PH group than in the preoperative NASH liver ($P$ < 0.05). On the other hand, fewer Ki-67-positive cells were noted in the NASH 70% PH group than in the preoperative NASH liver ($P$ < 0.01). Additionally, significantly fewer Ki-67-positive cells were noted in the 70% PH group than in the NASH 30% PH

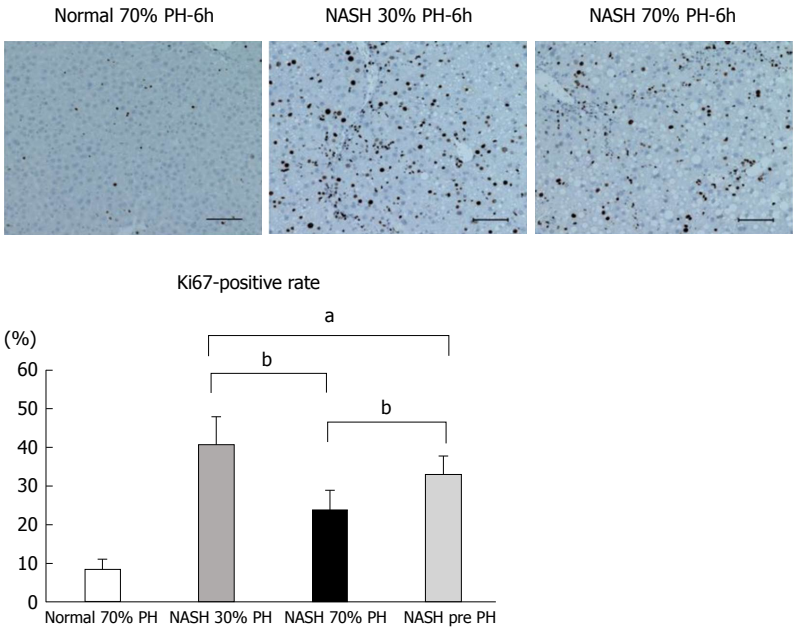


**Figure 2 Ki-67 staining and proliferation score.** Proliferation was evaluated by Ki-67 staining. We compared preoperative nonalcoholic steatohepatitis (NASH) groups with NASH 30% PH and NASH 70% PH groups 6 h after PH. Ratios of Ki-67-positive/total hepatocytes were calculated. Significantly fewer Ki-67-positive cells were noted in the 70% PH group than in the NASH 30% PH group. Ratios (%) are expressed as the mean ± SD. $n$ = 2 per groups, 10 fields per sample. $^{a}P < 0.05$; $^{b}P < 0.01$. Scale bar: 100 μm. PH: partial hepatectomy; NASH: nonalcoholic steatohepatitis.

group ($P < 0.01$) (Figure 2).

### *Liver regeneration signal*

In the normal 70% PH liver group, *i.e.*, the control group, expression of AKT, STAT3, and ERK1/2 phosphorylation was observed. In the both NASH 30% PH and 70% groups, phosphorylation of AKT, STAT3, and ERK1/2 was significantly lower than in the control group ($P < 0.01$) (Figure 3).

### *Histological assay*

The number of TUNEL-positive cells in the NASH 70% PH group was significantly higher than in the other groups ($P < 0.01$). The TUNEL-positive rate of normal liver was significantly higher than that in the NASH 30% PH group ($P < 0.01$) (Figure 4A). The area of necrosis in the NASH 70% PH group was significantly larger than that in the NASH 30% PH group ($P < 0.01$). In both NASH groups, the necrotic area was significantly larger than that in the normal liver group ($P < 0.01$) (Figure 4B).

### *Microarray assay*

mRNAs in the NASH 70% PH group with the highest fold-change (> 1.4 or < 0.70) in expression and with *P*-values <0.05 were selected and compared with those in the NASH 30% PH group (Table 2). *PDE4B*, *SLC20A1*, *CXADR*, *GADD45A*, *ZSWIM6*, and *C15orf39* were expressed at higher levels in the NASH 70% PH group. *PDE4B* and *GADD45A* are associated with cell cycle arrest and apoptosis. Using qPCR, *GADD45A* and *PDE4B* mRNA expression was significantly different between the two groups (*GADD45A*: $P < 0.01$, *PDE4B*: $P < 0.05$) (Figure 5).

## DISCUSSION

NAFLD/NASH is a common hepatic disorder that causes HCC[1-4]. Recently, the population of patients with NASH and NASH-related HCC has been increasing[1,2,10]. Hepatectomy is the first-line treatment for patients with HCC[21]. After hepatectomy, the incidences of mortality and morbidity are dependent on the volume and function of the residual liver[17-20]. Previous reports have demonstrated that steatosis impaired liver regeneration and caused liver dysfunction after hepatectomy[11,12]. NASH has been proposed to cause liver failure rather than steatosis because NASH presents with not only steatosis but also fibrosis, inflammation, and insulin resistance. However, regarding NASH animal models, few models completely reflect the histopathology and pathophysiology of NASH in humans. Therefore, the effect on the residual liver under NASH conditions has not been appropriately evaluated[26,27]. In our previous study, we established a novel experimental NASH model that exhibited histopathological and pathophysiological findings similar to that of NASH in humans[28]. In this study, new NASH mice were received 30% PH or 70% PH, and the influence of liver resection volume on the residual liver function in NASH liver was investigated. Our results indicated that the survival rate after PH in NASH liver strongly correlated with resected liver volume and was attributed to the proliferative ability and the rates of apoptosis and necrosis compared

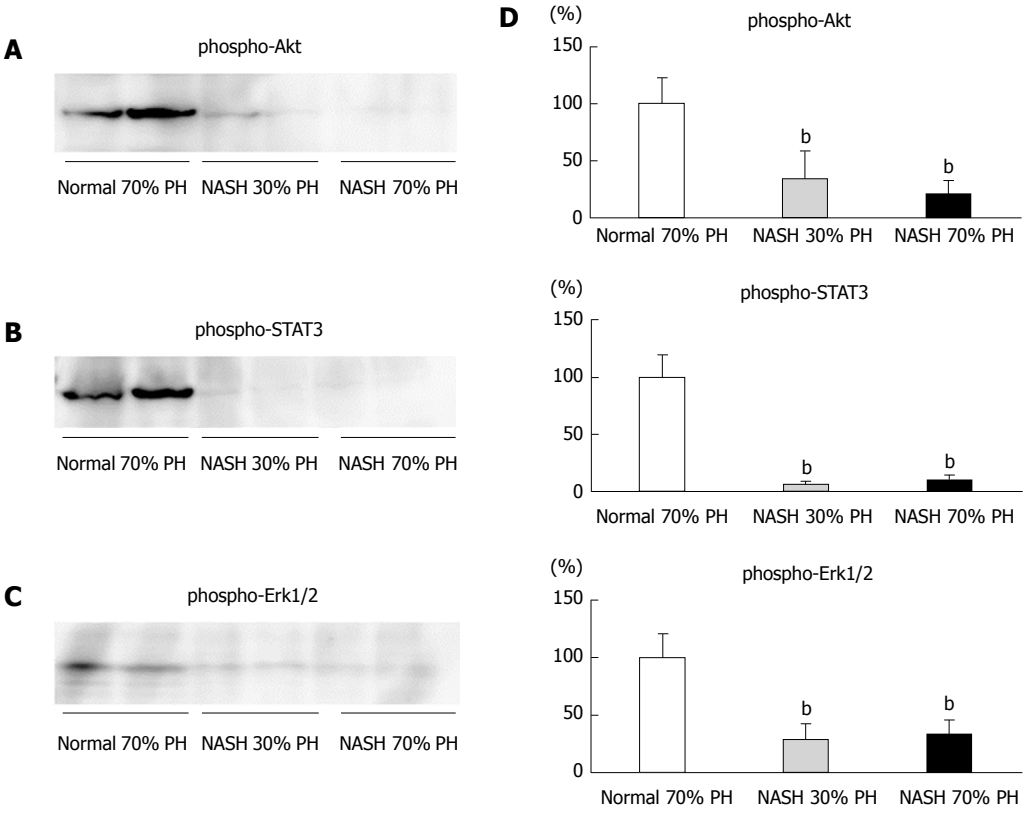


**Figure 3 Protein assay.** Expression of phosphorylated (A) Akt, (B) STAT3, and (C) ERK1/2 in normal and nonalcoholic steatohepatitis liver groups 6 h after PH. Expression levels were detected by western blot analysis. Ratios of densitometry were calculated by each score/score of control 70% PH (D). Data are expressed as the mean ± SD. $n$ = 5-7 per groups. $^{b}P < 0.01$ *vs* other groups.

with those in the normal liver. Even 30% of PH NASH residual liver could not offer sufficient liver function, and the volume of the functional residual liver significantly decreased due to less cell proliferation, apoptosis and necrosis after hepatectomy. Based on these results, we hypothesized that the residual liver volume that can support sufficient function in a normal liver could not maintain liver function in NASH. Our results suggested that to avoid liver dysfunction after hepatectomy in NASH, resection volume should be carefully determined and not the same as that in patients with normal liver.

In patients with PH, fatty liver causes a high rate of mortality and morbidity compared with normal liver[11]. In NAFLD patients, postoperative complications also increase in a manner that is similar to patients with fatty liver[12,30]. In animal models with PH, the survival rate of a fatty liver model decreased compared with a normal liver even with the same residual volume[13,15,16,31]. In this study, the survival rate after PH remarkably decreased in the NASH 70% PH group, and 30% of the deaths occurred within the first 48 h after PH. This result supported the previous reports, *i.e.*, outcome of PH significantly influences the survival rate of fatty mice[13,15,16,31]. In NASH liver, it was assumed that other characteristics, *i.e.*, fibrosis, inflammation, and insulin resistance, caused increased liver function deterioration. It was hypothesized that the residual liver volume of the small group, *i.e.*, 30% of residual volume of the NASH liver, could not maintain sufficient liver function for survival after PH.

Liver regeneration occurred in cases with acute injury and/or liver resection[19]. In normal liver after PH, cell proliferation was observed in a small residual liver but not in a large residual liver[24]. Large residual livers have sufficient volume to maintain liver function, whereas small residual livers are unable to maintain liver function. Therefore, promotion of cell proliferation occurs in the small residual liver[24]. Ki-67 protein is expressed during the G1, G2, and S phases of cell division[32,33]. In this study, the number of Ki-67-positive cells in the residual liver in the 30% PH of NASH liver group was higher than in the preoperative NASH liver. On the other hand, the number of Ki-67-positive cells in the residual liver of the 70% PH of NASH liver group was significantly decreased. These results suggested that NASH hepatocytes would not have insufficient proliferation ability after large amount of PH, such as 70%.

Signaling pathways of liver regeneration are promoted by cytokines, *i.e.*, interleukin (IL)-6 and tumor necrosis factor (TNF)-alpha, and growth factors[20]. The expression of AKT, STAT3, and ERK1/2 protein play an important role in liver regeneration, and the IL-6/STAT3 signaling pathway accelerates liver proliferation[16,24,34-36]. STAT3 was expressed at high levels in a liver with steatosis; however, these phenomena did not induce liver regeneration[37]. The NASH liver received continuous

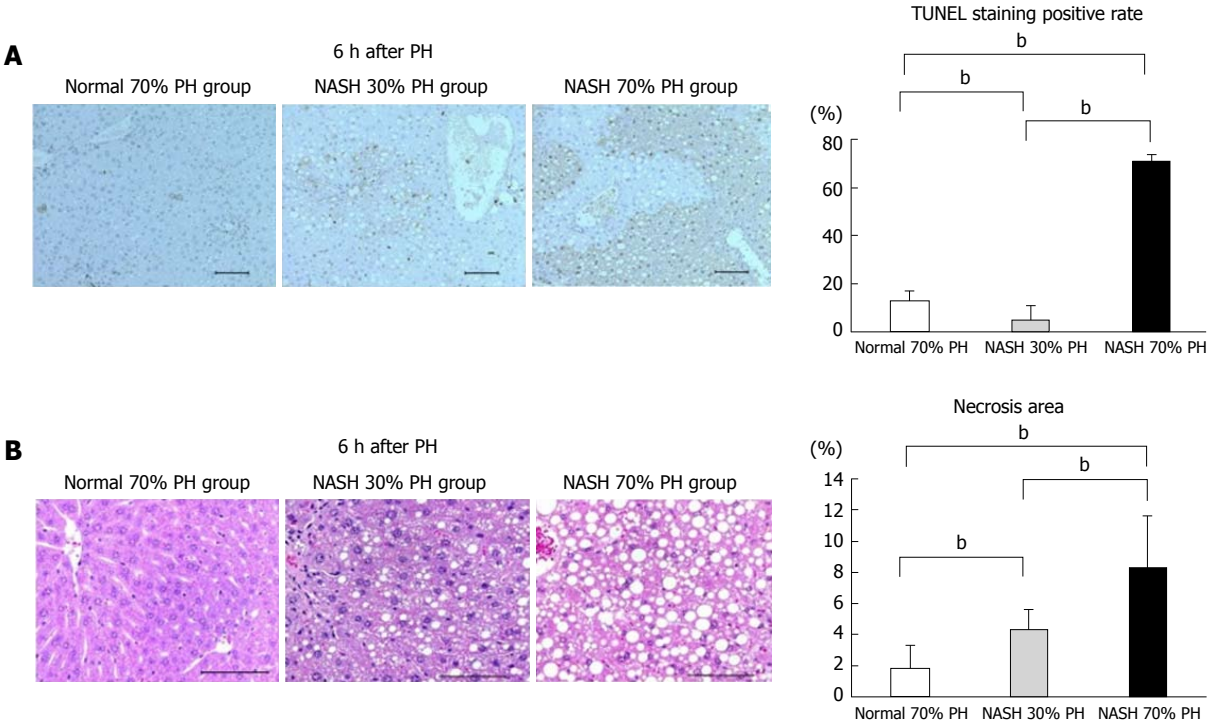


**Figure 4 Histopathological findings.** A: Apoptosis was evaluated by TUNEL staining. Ratios of TUNEL-positive/total hepatocytes were calculated; B: Necrosis was evaluated by HE staining. Ratios of necrosis morphological feature/total area were calculated. Apoptosis and necrosis were higher in the NASH 70% PH group than in the other groups. Data are expressed as the mean ± SD. $n$ = 2 per groups, 10 fields per sample. $^{b}P < 0.01$. Scale bar: 100 μm. PH: Partial hepatectomy; NASH: Nonalcoholic steatohepatitis.

**Table 2 Gene expression microarray**

| Function | Gene name | Gene abbreviation | Fold-change (> 1.4) | *P* value (< 0.05) |
|---|---|---|---|---|
| Regulate the cellular concentrations of cyclic nucleotides | Phosphodiesterase 4B, cAMP-specific | *PDE4B* | 2.102691211 | 0.0063 |
| Growth arrest | Growth arrest and DNA-damage-inducible, alpha | *GADD45A* | 1.672778704 | 0.013 |
| Signal transduction | Coronin 1C | *CORO1C* | 1.489851976 | 0.031 |
| Stimulates expression of cytokines, including IL6, MIF and VEGFA | Hypoxia inducible lipid droplet associated | *HILPDA* | 1.481465541 | 0.011 |
| EGF-like growth factor | Heparin binding EGF-like growth factor | *HBEGF* | 1.432049736 | 0.043 |
| Cell-cell junctions | Membrane associated guanylate kinase, WW And PDZ domain containing 1 | *MAGI1* | 1.430522907 | 0.0222 |
| Innate immune system | Ankyrin repeat and SOCS box containing 13 | *ASB13* | 0.515175325 | 0.0042 |
| Apoptosis and autophagy | TIA1 cytotoxic granule-associated RNA binding protein-like 1 | *TIAL1* | 0.609948905 | 0.027 |
| Gene expression | Nucleic acid binding protein 1 | *NABP1* | 0.6558797325 | 0.042 |
| Cell cycle | S-phase kinase-associated protein 2, E3 ubiquitin protein ligase | *SKP2* | 0.6588204077 | 0.044 |
| Mitochondrial metabolism | Translocase of inner mitochondrial membrane 9 homolog (yeast) | *TIMM9* | 0.6598542503 | 0.042 |
| Cytokine signaling in immune system | B-cell CLL/lymphoma 6 | *BCL6* | 0.6760241074 | 0.0097 |
| Cell cycle | Mutated in colorectal cancers | *MCC* | 0.6761191711 | 0.0076 |
| Gene expression | Zinc finger protein 519 | *ZNF519* | 0.6864230003 | 0.028 |
| Gene expression | RNA binding motif protein, X-linked | *RBMX* | 0.6983542235 | 0.012 |

In total, 6 genes were overexpressed greater than 1.4-fold in the nonalcoholic steatohepatitis (NASH) 70% PH group compared with their expression in the NASH 30% PH group, and 7 genes were downregulated by less than 0.70-fold in the NASH PH group compared with their expression in the NASH 30% PH group ($n$ = 2). PH: Partial hepatectomy.

damage and stress *via* inflammatory cytokines and cells; therefore, NASH liver was considered to exhibit limited proliferation, which was only induced by hepatectomy[38]. In this study, the expression of transcriptional factors induced by cytokines for liver regeneration was not recognized in the residual livers of the NASH PH groups. No significant difference was noted in the activation of these proteins in both NASH PH groups, but liver cell proliferation was significantly higher in the 30% PH NASH liver group than in the 70% PH NASH liver group.

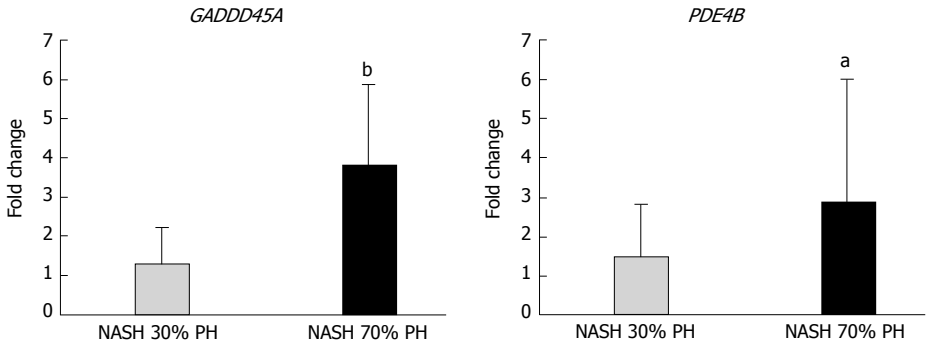


**Figure 5 Gene expression of microarray.** *GADD45A* and *PDE4B* expression correlated with the cell cycle. RT-PCR demonstrated a significant difference between the groups. Data are expressed as the mean ± SD. $n$ = 5-7 per groups. [a]$P < 0.05$; [b]$P < 0.01$. PH: partial hepatectomy; NASH: nonalcoholic steatohepatitis.

Although we need to confirm these findings in future studies, the consistent decrease in liver proliferation exists, especially in the 70% PH of NASH liver group, *i.e.*, a large hepatectomy volume, which reduces the survival rate.

In general, the stress of liver resection promotes apoptosis in the residual liver[13,39], and liver damage after hepatectomy is considered to be the result of apoptosis to some degree[38]. The degree of liver damage also depends on the extent of the liver resection volume[37,39]. The STAT3 and AKT signaling pathways not only promote liver regeneration but also inhibit apoptosis[38]. In this study, STAT3 and AKT expression was significantly suppressed, and the number of TUNEL-positive cells was higher in the NASH PH groups than in the control. These results suggest that the difference in the expression of regenerative signaling proteins affected the degree of apoptosis. Resection of a large volume of the liver also enhanced necrosis[38]. In this study, microarray analysis revealed *GADD45A* upregulation in the NASH 70% PH group. *GADD45A* promotes apoptosis and cell cycle arrest[13]. The differences in the survival rate between 30% or 70% PH in the NASH groups are inversely proportional to the incidence of Ki-67-positive cells, apoptosis, and necrosis. *GADD45A* upregulation correlates with the differences between the NASH groups and the low survival rate in small residual NASH liver after 70% PH.

In conclusion, residual NASH liver dysfunction after hepatectomy is attributed to a reduction in liver regeneration and cell proliferation. These findings suggest that the resection volume is a more limiting factor in patients with NASH than in those with a normal liver. Regarding liver surgery, the risk of complications for patients diagnosed with NASH by liver biopsy should be determined before hepatectomy. Further studies are needed to clarify therapeutic agents for NASH using our novel NASH model.

## ARTICLE HIGHLIGHTS

### Research background

The population of patients with nonalcoholic steatohepatitis (NASH) and NASH-related hepatocellular carcinoma (HCC) has been increasing. However, few animal models fully reflect both the histopathology and pathophysiology of NASH in humans, therefore, the metabolism of the residual liver after a hepatectomy with NASH has not been clarified. We succeeded to establish a novel experimental NASH model that had same characteristics of histopathology and pathophysiology of NASH in humans.

### Research motivation

In NASH, continuous inflammation contributes to HCC. The cause of HCC is frequently infection with hepatitis B virus and hepatitis C virus (HCV). New antiviral medications for hepatitis are currently being used in clinics; therefore, the number of patients with virus-related HCC is expected to decrease in the future. By contrast, the number of patients with NASH-related HCC has been increasing recently, and this trend is expected to continue because no effective treatments are available

### Research objectives

The aim of this study was to investigate whether a difference in liver resection volume in a novel NASH model influences surgical outcomes.

### Research methods

To establishment of a NASH model, mice were fed a high-fat diet for 4 wk, administered $CCl_4$ for the last 2 wk and administered T0901317 for the last 5 d. These mice were divided into two groups: A 30% partial hepatectomy (PH) of NASH liver group and a 70% PH of NASH liver group (control). Evaluate the survival rate, regeneration, apoptosis, necrosis and DNA expression level after PH.

### Research results

In the 70% PH of NASH group, the survival rate was significantly decreased compared with that in the control and 30% PH of NASH groups ($P < 0.01$). 10 of 32 mice in the NASH 70% PH group died within 48 h after PH. serum aspartate aminotransferase (AST) levels and total bilirubin (T-Bil) in the NASH 70% PH group were significantly higher than the levels in the other two groups (AST: $P < 0.05$, T-Bil: $P < 0.01$). In both PH of NASH groups, signaling proteins involved in regeneration were expressed at lower levels than those in the control group ($P < 0.01$). The 70% PH of NASH group also exhibited a lower number of Ki-67-positive cells and higher rates of apoptosis and necrosis than the NASH 30% PH group ($P < 0.01$). In addition, DNA microarray assays showed differences in gene expression associated with cell cycle arrest and apoptosis.

### Research conclusions

The residual NASH liver dysfunction after hepatectomy is attributed to a reduction in liver regeneration and cell proliferation. A larger residual volume is required to maintain liver functions in mice with NASH.

### Research perspectives

This study suggests that the resection volume is a more limiting factor in patients with NASH than in those with a normal liver. Regarding liver surgery, the risk of complications for patients diagnosed with NASH by liver biopsy

should be determined before hepatectomy. Further studies are needed to clarify therapeutic agents for NASH using our novel NASH model.

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**P- Reviewer**: Lin GM, Tarantino G, Ulasoglu C **S- Editor**: Wang XJ
**L- Editor**: A **E- Editor**: Huang Y

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

*World J Gastroenterol* 2018 April 21; 24(15): 1632-1640

DOI: 10.3748/wjg.v24.i15.1632

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Retrospective Study

# Endoscopic submucosal dissection for early esophageal neoplasms using the stag beetle knife

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Author contributions: Kuwai T designed the research; Yamaguchi T, Imagawa H, Yamaguchi A, Kouno H, Kohno H and Ishaq S contributed by providing critical intellectual input for the revised manuscript; Kuwai T, Miura R, Sumida Y, Takasago T, Miyasako Y, Nishimura T and Iio S analyzed the data; Kuwai T wrote the article.

Institutional review board statement: This study was approved by the National Hospital Organization Kure Medical Center and the Chugoku Cancer Center Institutional Review Board Ethics Committee on 3 October 2016, the study incorporated good clinical practice, conforming to Declaration of Helsinki principles.

Informed consent statement: All patients were informed of the risks and benefits of ESD and provided written informed consent.

Conflict-of-interest statement: All authors declare no conflicts-of-interest related to this article.

Data sharing statement: No additional data are available.



Manuscript source: Invited manuscript

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Received: February 9, 2018

Peer-review started: February 11, 2018

First decision: March 9, 2018

Revised: March 16, 2018

Accepted: March 31, 2018

Article in press: March 30, 2018

Published online: April 21, 2018

## Abstract

### *AIM*

To determine short- and long-term outcomes of endoscopic submucosal dissection (ESD) using the stag beetle (SB) knife, a scissor-shaped device.

### *METHODS*

Seventy consecutive patients with 96 early esophageal neoplasms, who underwent ESD using a SB knife at

Kure Medical Center and Chugoku Cancer Center, Japan, between April 2010 and August 2016, were retrospectively evaluated. Clinicopathological characteristics of lesions and procedural adverse events were assessed. Therapeutic success was evaluated on the basis of *en bloc*, histologically complete, and curative or non-curative resection rates. Overall and tumor-specific survival, local or distant recurrence, and 3- and 5-year cumulative overall metachronous cancer rates were also assessed.

***RESULTS***

Eligible patients had dysplasia/intraepithelial neoplasia (22%) or early cancers (squamous cell carcinoma, 78%). The median procedural time was 60 min and on average, the lesions measured 24 mm in diameter, yielding 33-mm tissue defects. The *en bloc* resection rate was 100%, with 95% and 81% of dissections deemed histologically complete and curative, respectively. All procedures were completed without accidental incisions/perforations or delayed bleeding. During follow-up (mean, 35 ± 23 mo), no local recurrences or metastases were observed. The 3- and 5-year survival rates were 83% and 70%, respectively, with corresponding rates of 85% and 75% for curative resections and 74% and 49% for non-curative resections. The 3- and 5-year cumulative rates of metachronous cancer in the patients with curative resections were 14% and 26%, respectively.

***CONCLUSION***

ESD procedures using the SB knife are feasible, safe, and effective for treating early esophageal neoplasms, yielding favorable short- and long-term outcomes.

**Key words:** Neoplasms; Stag beetle knife; Esophageal; Endoscopic submucosal dissection;Outcome measures



**Core tip:** Various devices designed for endoscopic submucosal dissection (ESD) are currently under investigation for their usefulness in the treatment of early esophageal neoplasms. This study aimed to evaluate the short- and long-term outcomes of ESD using the stag beetle (SB) knife, a scissor-shaped device. Seventy-four patients with 101 esophageal lesions underwent resection via SB-knife ESD. Rates of en bloc, histologically complete, and curative resections were 100%, 95%, and 81%, respectively. The 3- and 5-year survival rates were 83% and 70%, respectively. The SB knife allows safe and effective ESD of early esophageal neoplasms.

Kuwai T, Yamaguchi T, Imagawa H, Miura R, Sumida Y, Takasago T, Miyasako Y, Nishimura T, Iio S, Yamaguchi A, Kouno H, Kohno H, Ishaq S. Endoscopic submucosal dissection for early esophageal neoplasms using the stag beetle knife. World J Gastroenterol 2018; 24(15): 1632-1640 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i15/1632.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i15.1632

## INTRODUCTION

Esophageal carcinoma is the eighth most common cancer worldwide and the sixth leading cause of cancer-related deaths globally[1,2]. Squamous cell carcinoma (SCC) is the commonest histotype of esophageal cancer in Japan and worldwide[1,3]. Despite advances in diagnosis and treatment, outcomes in these patients remain poor, with five-year survival rates of 15%-20%[4,5]. Aggressive use of enhanced imaging for screening and advanced magnification endoscopy systems have aided in early diagnosis. However, given the many possible comorbidities in patients undergoing conventional treatments (such as esophagectomy) and the greater likelihood of incomplete resection through endoscopic mucosal resection, researchers are now actively investigating endoscopic submucosal dissection (ESD) of superficial esophageal neoplasms[6-8].

A number of conventional ESD devices (*i.e.*, dual, flush, insulated-tip, and hook knives) have been utilized for esophageal ESD[9-14]. Compared to those of the stomach, the thin wall (with no serosa) and narrow lumen of the esophagus make ESD inherently more challenging. The endoscopic maneuverability difficulties imposed by conventional ESD devices are also problematic, particularly the lack of fixation to targets and the fact that these devices are partially or entirely uninsulated. Constraints of this sort are conducive to unintentional incisions, increasing the potential risk of adverse events such as perforation and mediastinal emphysema[15-18].

On the other hand, the stag beetle (SB) knife (Sumitomo Bakelite Co., Ltd., Akita, Japan), with its ability to grasp, assess, and then cut targeted tissue, allows endoscopists to maintain adequate dissection planes, preventing inadvertent injury to the muscular layer and promoting safe ESD[19-23]. Although early experiences at selected institutions suggest that the SB knife is safe and effective, no large series of patients or long-term outcomes have been reported to date[21-24]. The aim of this study was to investigate use of the SB knife for ESD of early esophageal neoplasms, assessing both feasibility and safety. Subsequent short- and long-term clinical outcomes were examined as well.

## MATERIALS AND METHODS

### *Study design*

A single-center retrospective review of collected data was conducted, examining 74 consecutive patients with 101 esophageal lesions who underwent resection *via* SB-knife ESD between April 2010 and August 2016 at Kure Medical Center and Chugoku Cancer Center,

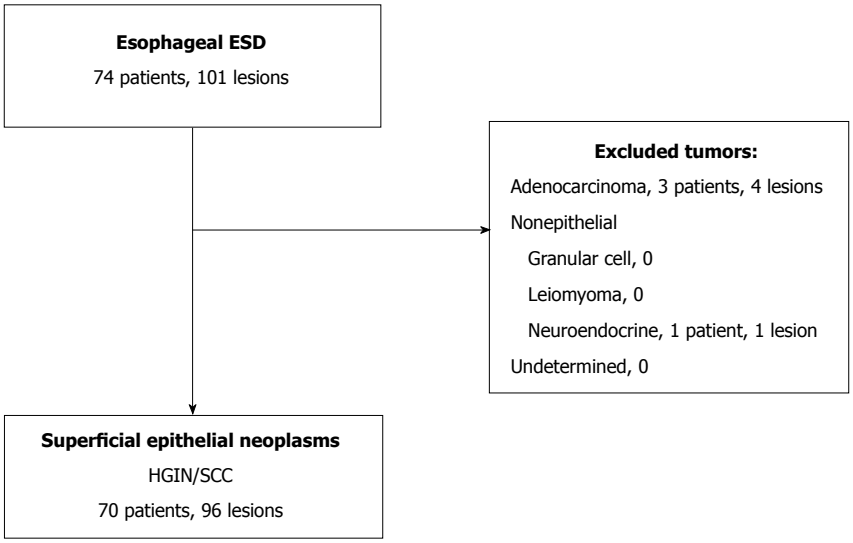


**Figure 1 Study design, evaluating the use of the stag beetle knife for esophageal endoscopic submucosal dissection.** ESD: Endoscopic submucosal dissection; HGIN: High-grade intraepithelial neoplasia; SCC: Squamous cell carcinoma.

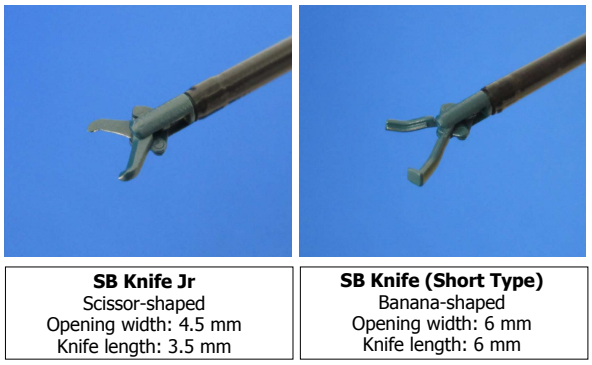


**Figure 2 Features of the stag beetle Knife Jr and short devices.**

Japan (Figure 1). All patients underwent resection using only SB-knife ESD during this time period. Approved by the National Hospital Organization Kure Medical Center and the Chugoku Cancer Center Institutional Review Board Ethics Committee on 3 October 2016, the study incorporated good clinical practice, conforming to Declaration of Helsinki principles. All lesions were diagnosed preoperatively during chromoendoscopy, identifying areas for biopsy through narrow-band imaging or iodine staining. Inclusion criteria were patients with superficial esophageal neoplasms (SENs), consisting of high-grade intraepithelial neoplasia or SCC. Exclusion criteria included patients with adenocarcinoma, non-epithelial tumors (*i.e.*, granular cell tumors, leiomyomas, and neuroendocrine tumors) or undetermined tumors.

All patients were informed of the risks and benefits of ESD and provided written informed consent. ESD was contraindicated in patients with serious comorbidities, distant metastasis, or massive submucosal invasion.

### *ESD procedure*

ESD procedures were performed by four board-certified endoscopists of the Japan Gastroenterological Endoscopy Society, three with no previous conventional esophageal ESD experience and one with low experience (10 cases). Patients received intravenous nitrazepam for sedation, and cardiorespiratory function was monitored throughout the procedure. A single-channel endoscope equipped with a water jet (GIF-H260Z; Olympus Corp, Tokyo, Japan) and attached transparent tip hood was routinely used, along with carbon dioxide insufflation. Initially, the outside margin of each lesion was marked using argon plasma coagulation in forced coagulation mode; the esophageal mucosa was injected with 0.4% sodium hyaluronate (MucoUp; Seikagaku Corp., Tokyo, Japan) mixed with a small amount of indigo carmine. Circumferential excision was then carried out with the SB Knife Jr (4.5-mm opening width, 3.5-mm length; Sumitomo Bakelite Co.) (Figure 2). For submucosal dissection, the SB Knife Short (6-mm opening width, 6-mm length) (Figure 2) was often preferred, because detachment/peeling of the submucosa was faster, and it was less likely to engage the muscular layer, given the curved shape of the blade. The SB knife allowed grasping of the targeted segment, which was then cut using a high-frequency generator (VIO300D; ERBE, Tubingen, Germany) in the endo-cut Q mode (effect 1) for incising mucosa and dissecting submucosa. The soft coagulation mode (effect 5.40 W) was used for hemostasis. If repeated coagulation was required, hemostatic forceps (Coagrasper; Olympus Corp.) were applied to facilitate endoscopic hemostasis. The procedure was continued until resection was completed (Figure 3).

In instances of semi-circumferential or circumferential ESD, intralesional diluted triamcinolone acetonide injected on postoperative day 2 [Kenacort (40-80 mg); Bristol-Myers Squibb Co., New York, NY, United States] or oral prednisolone (30 mg/d) was prescribed and tapered gradually over several weeks[25] to prevent

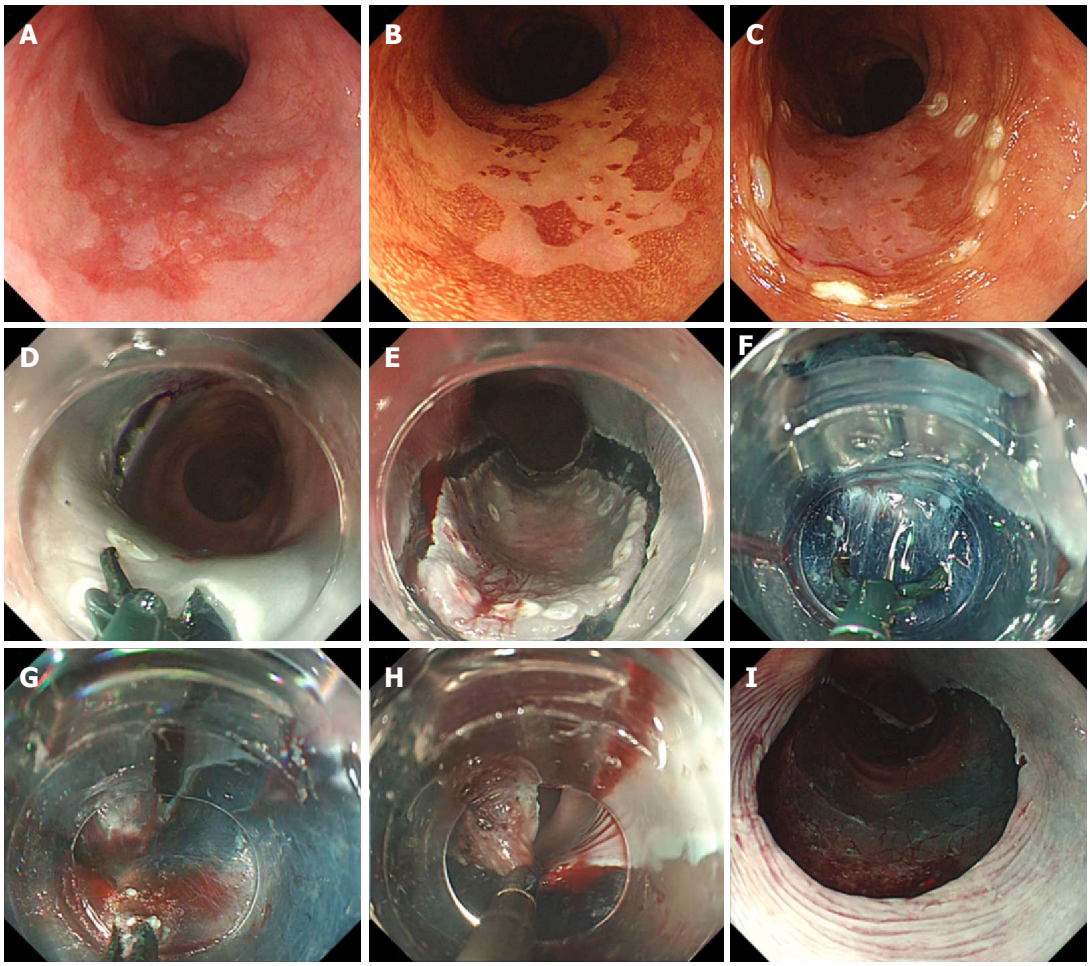


**Figure 3 Stag beetle knife used for esophageal endoscopic submucosal dissection in a 79-year-old man.** Endoscopic appearance of a 35-mm, depressed lesion in the middle one-third of the esophagus (A) under white light (B) on a scatter image with Lugol's iodine applied (C) with argon plasma coagulation markings; D and E: Use of the SB Knife Jr for full-circumferential incision; use of the SB Knife Short for (F) submucosal dissection and (G) hemostasis; H: *En bloc* resection of the lesion; I: Ulcer floor after resection.

postoperative stricture[26,27].

### Histopathology and short-term outcomes

Resected specimens were immediately fixed in 10% buffered formalin, with samples later selected for routine processing, embedding in paraffin, and slide preparation (3-4 μm, hematoxylin & eosin stain). The histotype, depth of invasion, and resection margins (vertical and lateral) of the lesions were assessed microscopically (Figure 4) using an optical micrometer to measure invasive areas. The tumor size, anatomic location (upper one-third, middle one-third, or lower one-third of the esophagus), and extent (%) of esophageal circumferential involvement were documented. Rates of *en bloc* resection, histologically complete resection (*i.e.*, *en bloc* resection with negative lateral and vertical margins), and curative or non-curative resection served as indices of therapeutic success. Curative resection was defined as complete tumor resection with invasion $\leqslant$ 200 μm below the deep border of the lamina muscularis mucosae and no lymphovascular involvement[8].

### Adverse events

Immediate adverse events such as perforation, delayed bleeding, and postoperative pneumonia and delayed adverse events such as esophageal stricture were recorded.

### Long-term outcomes

To monitor patients, esophagogastroduodenoscopy was performed 3-6 mo and 1 year following ESD and annually thereafter; computed tomography was also performed annually. Three- and 5-year overall survival rates were assessed for the entire study cohort. SENs detected > 1 year after curative resection by ESD were considered metachronous cancers. Cumulative overall metachronous cancer rates during the 3- and 5-year periods were also assessed.

### Statistical analysis

Continuous variables were expressed as a mean ± standard deviation or median and range, as appropriate, and categorical variables as frequency or number of

**Table 1 Study demographics and clinicopathologic characteristics *n* (%)**

| Characteristics | Value |
|---|---|
| Number of patients | 70 |
| Number of lesions | 96 |
| Age, mean ± SD (range), yr | 67 ± 10 (43-87) |
| Sex | |
| Male | 59 (84) |
| Female | 11 (16) |
| Location of the tumor in the esophagus | |
| Upper one-third | 11 (11) |
| Middle one-third | 53 (55) |
| Lower one-third | 33 (34) |
| Gross appearance | |
| Depressed | 86 (90) |
| Elevated | 7 (7) |
| Flat | 2 (2) |
| Mixed | 1 (1) |
| Resected specimen size, mean ± SD (range), mm | 33 ± 14 (9-75) |
| Resected tumor size, mean ± SD (range), mm | 24 ± 13 (1-64) |
| Luminal extent | |
| < 1/2 | 59 (61) |
| ⩾ 1/2, < 2/3 | 20 (21) |
| ⩾ 2/3 | 17 (18) |
| Histopathologic features | |
| Dysplasia/intraepithelial neoplasia | 21 (22) |
| Squamous cell carcinoma | 75 (78) |
| Epithelial lining | 15 (20) |
| Lamina propria mucosae | 31 (41) |
| Muscularis mucosae | 13 (17) |
| Submucosa (SM1) | 2 (3) |
| Submucosa (SM2 or deeper) | 14 (19) |

**Table 2 Short-term outcomes and adverse events of esophageal endoscopic submucosal dissection, *n* %**

| | 95%CI |
|---|---|
| Procedure duration, median (range) | 60 (25-305) |
| *En bloc* resection | 96 (100) [96.2-100] |
| Complete resection with negative margins | 91 (95) [88.4-97.8] |
| Curative resection | 78 (81) [72.3-87.8] |
| Adverse events | |
| Perforation | 0 (0) [0-3.9] |
| Delayed bleeding | 0 (0) [0-3.9] |
| Pneumonia | 3 (3) [1.1-8.8] |
| Esophageal stricture | 7 (7) [3.6-14.3] |

occurrences. Kaplan-Meier curves were generated to analyze survival and metachronous cancer rates. A log-rank test was used to evaluate the significance of differences between curves, and a *P* value of less than 5% was considered significant. All statistical analyses were performed using JMP software (SAS Institute, Inc., Cary, NC, United States).

## RESULTS

### Demographic and clinicopathologic characteristics

A total of 96 SENs in 70 patients qualified for analysis. One subject was excluded, having received a final diagnosis of nonepithelial tumor, and 4 subjects were excluded, having received a final diagnosis of adenocarcinoma (Figure 1). Fifteen patients had multiple lesions, harboring two (*n* = 9), three (*n* = 3), four (*n* = 1), or five (*n* = 2) lesions. Mean age of the study population (*n* = 70; 84% men) was 67 ± 10 years (Table 1). By location, 11% of lesions involved the upper one-third of the esophagus, 55% the middle one-third, and 34% the lower one-third. Macroscopically, majority of the lesions were depressed (90%) rather than elevated (7%) or flat (2%).

Resected specimens measured 33 ± 14 mm on average, with a mean tumor size of 24 ± 13 mm. Most tumors (61%) involved < one-half of the esophageal luminal circumference. Histopathological diagnoses were as follows: dysplasia/intraepithelial neoplasia (22%), or SCC (78%). Typically, invasive SCCs were limited to the lamina propria mucosae (41%), with SM2 or deeper infiltration accounting for 19% (Table 1).

### Short-term outcomes

Short-term outcomes are summarized in Table 2. The median procedural time was 60 min (range, 25-305 min). Rates of *en bloc* resection, histologically complete resection, and curative resection were 100%, 95%, and 81%, respectively.

### Adverse events

All lesions were safely resected without any unintentional incisions/perforations or delayed bleeding episodes. Pneumonia was observed in 3% of patients and managed through antibiotic treatment. To prevent postoperative stricture after semi-circumferential ESD, intralesional injection of triamcinolone acetonide (3 patients) or oral prednisolone (7 patients) was administered. Esophageal strictures were encountered in seven patients, one requiring balloon dilatation (Table 2).

### Long-term outcomes

Curative resection was achieved in 57 patients, three of whom received additional chemoradiotherapy (CRT). One of these three patients later developed metachronous cancer. Seven of the 54 patients who were given no additional treatment also developed metachronous cancers. The 3- and 5-year cumulative rates of metachronous cancer in patients with curative resections were 14% and 26%, respectively (Figure 5A). Non-curative resection was achieved in 13 patients, seven of whom underwent additional treatment, either surgery (*n* = 4), chemotherapy (*n* = 1), or CRT (*n* = 2). No instances of local recurrence or metastasis were observed in any patient during the mean follow-up period of 35 ± 23 mo.

### Survival analysis

Three- and 5-year overall survival rates for the study cohort were 83% and 70%, respectively (Figure 5B), with corresponding rates of 85% and 75% in curative resections, and 74% and 49% in non-curative resections (Figure 5C). However, the difference in survival between curative and non-curative resections

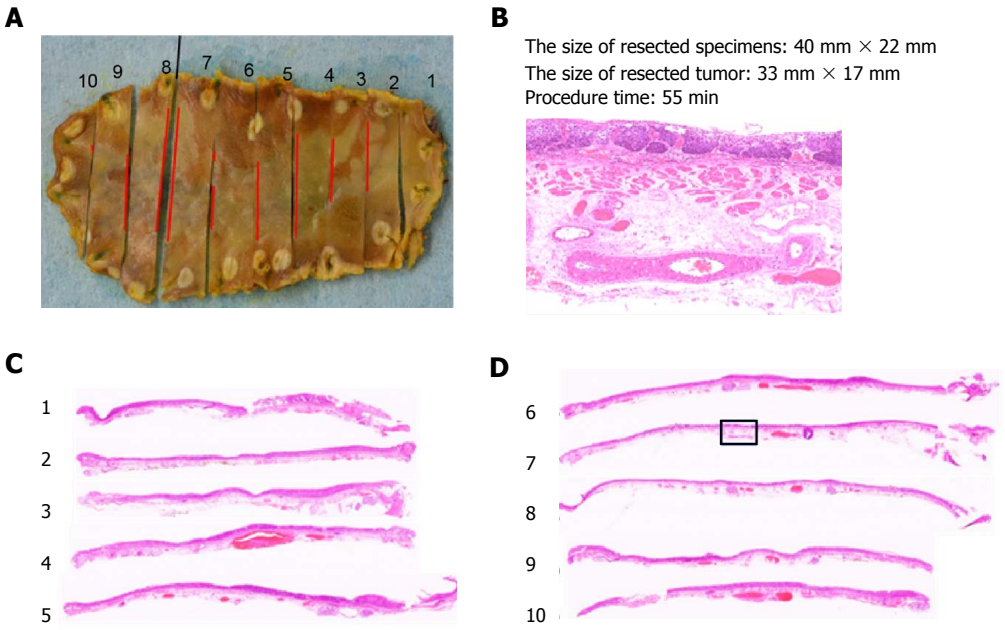


**Figure 4 Formalin-fixed specimen sliced at 2-mm intervals for routine processing and slide preparation (A); Evaluation of the histotype, invasion depth, and vertical/lateral resection margins (B-D).**

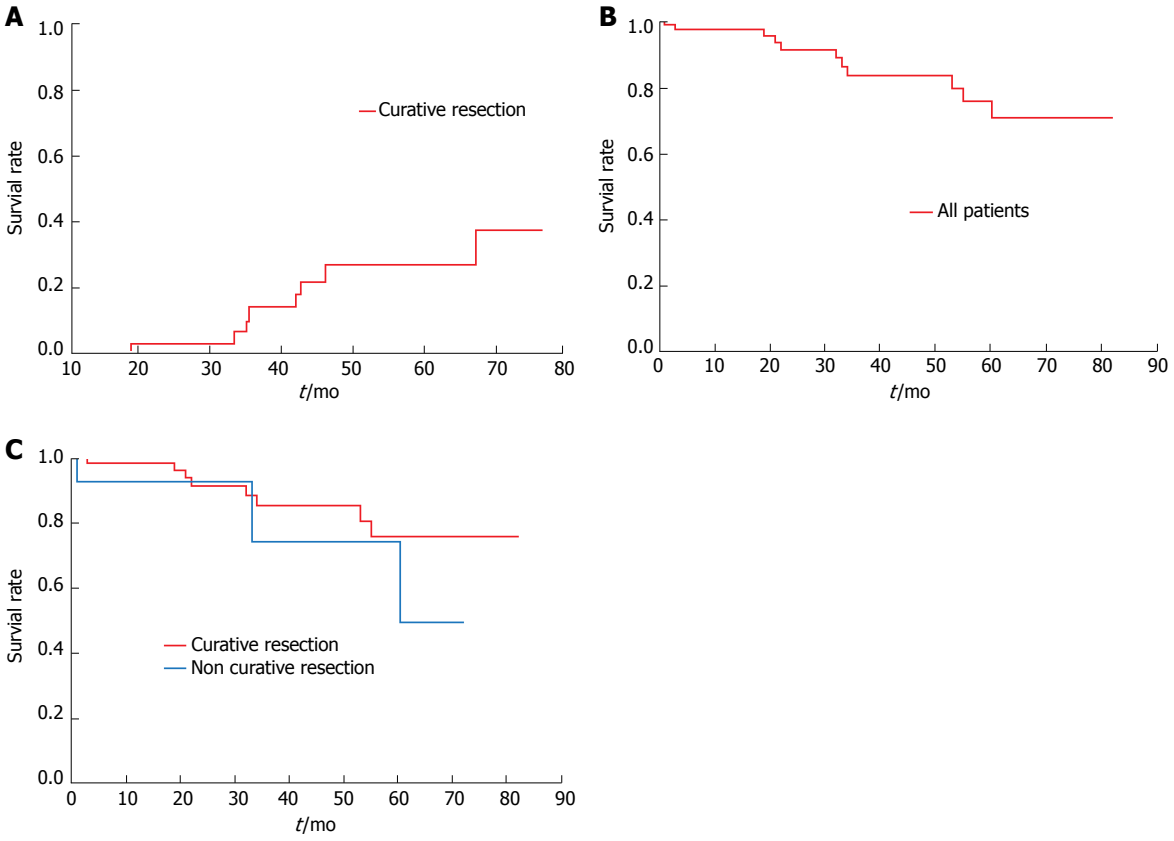


**Figure 5 Long-term outcomes and survival analysis.** A: Cumulative metachronous cancer rates in patients with curative resection; B: Kaplan-Meier analysis of overall survival rates in all patients; C: Patients grouped according to curative and non-curative resection.

by ESD was not statistically significant. Eleven of 70 patients (curative resections, 8/57; non-curative resections, 3/13) died during follow-up. In patients with curative resections, causes of death included isolated instances of unknown primary cancer, hepatocellular carcinoma, lung cancer, pharyngeal carcinoma, drowning, and aspiration pneumonia, as well as two instances of interstitial pneumonia. Causes of death after non-curative resection were oropharyngeal carcinoma, liver cirrhosis, and aspiration pneumonia,

each a single occurrence. None of the deaths was directly attributable to esophageal neoplasms.

## DISCUSSION

Our study suggests that the SB knife is safe and effective when used for ESD of early esophageal neoplasms. This technique resulted in high rates of *en bloc* resection (100%), histologically complete resection (95%), and curative resection (81%), with a low incidence of procedural adverse events, despite three of the certified endoscopists having either limited or no experience of esophageal ESD. Specifically, there were no unintentional incisions/perforations or delayed bleeding events. Furthermore, no patient experienced local recurrences or metastases in the long term, and the overall survival rates were highly favorable and similar to those for other devices[8,28,29].

Data reported here are consistent with those of a previous study demonstrating the safety and efficacy of the SB knife as a means of esophageal ESD in 15 patients[23]. Importantly, overall adverse events for the SB knife are relatively low[10-14]. Its unique scissor-action allows surgeons to grasp and pull the targeted tissue away from the muscularis and then inspect the area before cutting for more controlled resection and avoidance of perforation. Unlike other devices[30,31], no complex adjustments or special endoscopic techniques are required. This facilitated the acquisition of implementation skills by even general endoscopists[19,30] and shortened the training process[31-34], and we obtained good results from the first case itself. The notable absence of perforation reflects the safe and easy use of the SB knife in this setting, despite cyclic respiratory and cardiac fluctuations encountered during esophageal ESD. This tool also acts as a hemostatic clamp, eliminating the need for separate hemostatic forceps, making the procedure simple and cost-effective, and encouraging high resectability rates.

We would like to emphasize that no tumors recurred when the SB knife was used for esophageal ESD. Earlier studies have already reported positive short-term treatment outcomes (*i.e.*, resection rates) using the SB knife in small series[23,24]. The present results indicate for the first time, in the largest series reported to date, that use of the SB knife offers excellent short- and long-term outcomes in treating early esophageal neoplasms. The high resection rates achieved and absence of local recurrence or metastasis during 35 ± 23 mo of follow-up constitute a new paradigm shift in the treatment of SEN[14,16,17,28,35]. Consistent with other published studies[36], several of our patients with curative resections (8/57) developed metachronous lesions, including one of three who received additional CRT. This finding underscores the need for follow-up monitoring on a regular basis.

On analyzing longer-term patient outcomes, 3- and 5-year survival rates tended to be slightly, but not significantly, poorer if resections were non-curative (74% and 49%, respectively) rather than curative (85% and 75%, respectively). Although non-curatively resected esophageal cancer is typically associated with a poor prognosis, none of the deaths recorded in the present study were a direct result of esophageal cancer. The aforementioned trend may then have a singular explanation. As ESD ordinarily is not indicated in patients with SM-level invasive cancer, such patients who agreed to undergo ESD for excisional biopsy (the goal being localized control) were included in the non-curative resection group. Consequently, it appears that achieving local control of esophageal cancer *via* ESD is quite feasible in elderly patients and in those with serious underlying diseases, who ultimately may die from other causes. By seemingly benefitting from the safe performance of esophageal ESD, we have thereby demonstrated the efficacy of the SB knife in a high-risk population.

Contrary to earlier concerns that the use of the SB knife might prolong ESD procedures[16,18], we found that it greatly expedited ESD. The median time needed to complete ESD was 60 min (mean, 78 min), approximating the time required for conventionally performed ESD (median, 90 min) in one large multicenter study[8].

Our study has three limitations. It is retrospective, although data were collected from consecutive patients, it was conducted at a single center; and no comparison with another device was attempted. However, strengths of the study include the sizeable patient population and the extended follow-up period, both providing valuable data on the feasibility, safety, and efficacy of SB knife usage for esophageal ESD.

In conclusion, ESD procedures using the SB knife are feasible, safe, and effective for treating early esophageal neoplasms, yielding favorable short- and long-term outcomes. No perforation occurred in our study population, attesting to the innovative design of the SB knife, which allows better control for safer dissection. The availability of this tool may promote widespread adoption of ESD to treat early-stage cancers of the esophagus. There is need to conduct RCT studies to compare this new innovative device with established devices.

## ARTICLE HIGHLIGHTS

### *Research background*

Several conventional endoscopic submucosal dissection (ESD) devices have been utilized for esophageal ESD. The thin wall with no serosa and narrow lumen of the esophageal wall make ESD more challenging in the esophagus. The restricted endoscopic maneuvering required with conventional ESD devices is also problematic owing to lack of fixation to targets and the fact that these devices are partially or entirely uninsulated. These factors can lead to unintentional incisions, increasing the potential risk of adverse events such as perforation and mediastinal emphysema.

### *Research motivation*

The stag beetle (SB) knife, with its ability to grasp, assess, and then cut the targeted tissue allows endoscopists to maintain adequate dissection planes, preventing inadvertent injury to the muscular layer for safe ESD. Because of these advantages, the SB Knife is gaining acceptance, but relevant long-term

outcome data is limited.

### Research objectives

The aim of this study was to investigate use of the SB knife for ESD of early esophageal neoplasms, assessing both feasibility and safety. The subsequent short- and long-term clinical outcomes were examined as well.

### Research methods

We retrospectively reviewed 70 consecutive patients with 96 early esophageal neoplasms (HGIN/SCC) treated using ESD. An SB knife was used routinely in all procedures. Clinicopathologic characteristics of the lesions and rates of procedural adverse events, *en bloc* and histologically complete resection, overall and tumor-specific survival, and local or distant recurrence were assessed.

### Research results

The *en bloc* resection rate was 100%, with 95% and 81% of dissections deemed histologically complete and curative, respectively. All procedures were completed without accidental incisions/perforations or delayed bleeding. During follow-up (mean, 35 ± 23 mo), no local recurrences or metastases were observed. The 3- and 5-year survival rates were 83% and 70%, respectively. The 3- and 5-year cumulative rates of metachronous cancer in the patients with curative resections were 14% and 26%, respectively.

### Research conclusions

ESD procedures using the SB knife are feasible, safe, and effective for treating early esophageal neoplasms, yielding favorable short- and long-term outcomes. No perforation occurred in our study population, attesting to the innovative design of the SB knife, which allows better control for safer dissection.

### Research perspectives

The availability of this tool may promote widespread adoption of ESD to treat early-stage cancers of the esophagus. There is a need to conduct RCT studies to compare this new innovative device with established devices.

## ACKNOWLEDGMENTS

Writing support was provided by Cactus Communications. The authors extend thanks to Naoko Matsumoto for data collection and administrative assistance.

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**P- Reviewer**: Chetty R, Sugimoto M **S- Editor**: Wang XJ
**L- Editor**: A **E- Editor**: Huang Y

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

*World J Gastroenterol* 2018 April 21; 24(15): 1641-1649

DOI: 10.3748/wjg.v24.i15.1641

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Retrospective Study

# Analysis of aggressiveness factors in hepatocellular carcinoma patients undergoing transarterial chemoembolization

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Author contributions: All authors equally contributed to this manuscript.

Institutional review board statement: The Tel-Aviv medical center database management conforms to Israeli legislation on privacy and this study was approved by the institutional research committee in Tel-Aviv Medical Center (Approval number: 0528-16-TLV) in accordance with the ethical standards of the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent statement: Our manuscript is a retrospective study, therefore an informed consent waver was given by the IRB. Data was anonymized to prevent identification.

Conflict-of-interest statement: Professor Shibolet has nothing to disclose.

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at orensh@tlvmc.gov.il. Consent was not obtained but the presented data are anonymized and there is no risk of patient identification. The potential benefits of sharing these data outweigh the potential harms because of its possible application in improving future identification and treatment of HCC.



Manuscript source: Unsolicited manuscript

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Received: January 3, 2018
Peer-review started: January 4, 2018
First decision: January 16, 2018
Revised: March 10, 2018
Accepted: March 25, 2018
Article in press: March 25, 2018
Published online: April 21, 2018

## Abstract

### *AIM*

To investigate novel predictors of survival in hepatocellular carcinoma (HCC) patients following transarterial chemoembolization (TACE).

### *METHODS*

One hundred sixty seven patients with un-resectable HCC were retrospectively analyzed to identify factors that might contribute to their HCC biology and aggre-

ssiveness. We correlated routine laboratory results (total bilirubin, AST, ALKP, GGTP, albumin *etc*.) to maximum tumor diameter, number of tumor nodules, portal vein thrombosis and blood alpha-fetoprotein levels. These 4 parameters were previously combined to form an aggressiveness index (AgI). We used The Wilcoxon rank-sum (Mann-Whitney), to test the correlation between the AgI categories and liver function parameters. The Cox proportional hazards model was applied to evaluate the categories of AgI associated with overall survival.

***RESULTS***

The AgI was strongly correlated with survival in this novel patient population. Three year survival probability for AgI > or < 4 was 42.4% *vs* 61.8%; $P < 0.0863$ respectively. Several factors independently correlated with AgI using univariate multiple logistic regression of AgI with 8 laboratory parameters. Lower albumin levels had an OR of 2.56 (95%CI: 1.120-5.863 $P < 0.026$), elevated Alkaline phosphatase and gamma glutamyl transpeptidase (GGTP) had ORs of 1.01 (95%CI: 1.003-1.026, $P < 0.017$) and 0.99 (95%CI: 0.99-1.00, $P < 0.053$) respectively. In a Cox proportional hazard model combining mortality for AgI score and liver function parameters, only GGTP levels and the AgI were independently associated with survival. An AgI > 4 had HR for mortality of 2.18 (95%CI: 1.108-4.310, $P < 0.024$). GGTP's single unit change had a HR for mortality of 1.003 (95%CI: 1.001-1.006, $P < 0.016$). These were considered in the final multivariate model with the total cohort. An AgI > 4 had a HR for mortality of 2.26 (95%CI: 1.184-4.327, $P < 0.016$). GGTP had a HR of 1.003 (95%CI: 1.001-1.004, $P < 0.001$).

***CONCLUSION***

Our study validates the AgI in a new population with un-resectable HCC patients undergoing TACE. The analysis establishes a correlation between GGTP and the AgI.

**Key words:** Hepatocellular carcinoma; Aggressiveness index; Liver function; Transarterial chemoembolization; Survival



**Core tip:** Our cohort's population included patients with multiple underlying liver diseases and can be widely generalized. The aggressiveness index (AgI) was correlated with survival. AgI > 4 was associated with decreased survival. Combining the AgI with elevated GGTP and ALKP levels improved its prognostic yield in our patient population. We validated the AgI as a prognostic tool to predict overall survival in a novel population of hepatocellular carcinoma patients undergoing transarterial chemoembolization.

Ventura Y, Carr BI, Kori I, Guerra V, Shibolet O. Analysis of aggressiveness factors in hepatocellular carcinoma patients undergoing transarterial chemoembolization. *World J Gastroenterol* 2018; 24(15): 1641-1649 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i15/1641.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i15.1641

## INTRODUCTION

Hepatocellular carcinoma (HCC) is the fourth most common cancer and the third leading cause of cancer-related deaths in the world[1]. In the last several decades the incidence of HCC in developed countries has been rising, secondary to an increased incidence of HCV and non alcoholic steatohepatitis (NASH) associated cirrhosis[2]. The annual Nation Cancer Report of the United States published in 2016, noted that between 2003 and 2012, in contrast to the general decline in cancer incidence rates, HCC incidence continues to rise. Most cases of HCC arise on the background of chronic liver disease. Patients are usually asymptomatic until late in their disease, when symptoms and signs related to their cirrhosis are manifested. Early detection of HCC can be accomplished by screening populations at risk. The recommended surveillance of cirrhotic patients is abdominal ultrasound every six-months[3]. Measuring levels of alpha fetoprotein (AFP) a serum marker for HCC can be used together with ultrasonography. However, due to its low sensitivity and specificity it was recently omitted from clinical practice guidelines[4]. A staging system introduced by the Barcelona Clinic Liver Cancer (BCLC), is currently recommended as the best method for HCC staging and treatment allocation. The system incorporates the dimensions of the primary lesion, vascular invasion, extra-hepatic spread, performance status, general symptoms and the degree of severity of the underlying liver disease according to the Child-Pugh-Turcot score[5].

Trans-arterial chemoembolization (TACE) is performed by catheterization of tumor feeding branches of the hepatic artery and injecting chemotherapy with Lipidol. After the injection, the artery is embolized by particles. The TACE procedure is the treatment of choice for non-operable, intermediate stage HCC according to the BCLC classification[6]. Survival after the procedure varies and ranges between 12 to 34 mo[7]. Given the complexity of TACE and the variability in response, there is an urgent need to identify prognostic indices to predict overall survival in HCC patients undergoing the procedure[8]. Current prognostic indices include different inflammation scores such as the Glasgow prognostic score (GPS), neutrophil to lymphocyte ratio (NLR) and staging systems such as Barcelona Clinic Liver Cancer (BCLC), and Cancer of the Liver Italian Program (CLIP) scores. The GPS score was demonstrated as an independent marker of poor prognosis in patients with HCC and as a prognostic score predicting survival for patients with HBV related HCC after TACE[9-14]. All these indices have their shortcomings with some lacking strong

prognostic power (even when combined), and others lacking validation and or limited to specific populations.

An "HCC Aggressiveness" scoring system was recently described, which incorporates 4 tumor-related parameters: maximum tumor diameter (MTD), number of tumor nodules, portal vein thrombosis (PVT) and serum AFP levels. The score was shown to predict survival in HCC patients[15-17].

We retrospectively analyzed laboratory and clinical data from 167 patients with HCC that underwent TACE in Tel-Aviv medical Center in order to identify novel biomarkers to predict survival following TACE. These, 167 patients, were diagnosed predominantly through screening and thus at an earlier stage in their disease and some underwent the procedure as bridging for transplantation.

## MATERIALS AND METHODS

### *Patients and data collection*

We retrospectively analyzed prospectively-collected data from manual and computerized medical records of 167 HCC patients at Tel-Aviv Medical center, a tertiary center with a liver transplantation service, who underwent the TACE procedure between the years 2000 and 2015. We excluded patients with fibrolamellar HCC, mixed cholangio-hepatocellular carcinoma and sarcomatous type HCC.

Data was collected for 161 patients (6 patients were excluded because of missing data) during the 3 mo period before the first TACE procedure. Baseline tumor parameters including: maximum tumor diameter, number of tumor nodules and presence of PVT - were gathered from imaging reports carried out at Tel-Aviv medical center. Labs including: blood count; routine liver function tests, (total bilirubin, AST, ALKP, GGTP, albumin) and plasma AFP levels; demographics and overall survival information. The Tel-Aviv medical center database management conforms to Israeli legislation on privacy and this study was approved by the institutional research committee in Tel-Aviv Medical Center (Approval number: 0528-16-TLV) in accordance with the ethical standards of the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. We collected data to conform to the previously described aggressiveness index (AgI): including the following four parameters: Maximum Tumor dimension, AFP, presence or absence of PVT, and the number of tumor nodules. The AgI score was calculated as follows: MTD (in tertiles): MTD < 4.5; 4.5 $\leqslant$ MTD $\leqslant$ 9.6; MTD > 9.6; scores 1, 2, 3 respectively. AFP (cut-off): AFP < 100; 100 $\leqslant$ AFP $\leqslant$ 1000; AFP > 1000 ng/mL; scores 1, 2, 3 respectively. PVT (No/Yes): PVT (No); PVT (Yes); scores 1, 3 respectively. Tumor Nodule (number): Nodules $\leqslant$ 3; Nodules > 3; scores 1, 3 respectively. The AgI score was divided into three categories for Cox analysis (Table 1): a, score - < 4; b, 4 < score $\leqslant$ 7; and c, score $\geqslant$ 8.

### *TACE technique*

The TACE procedure was first introduced in 1974 by Doyon *et al*[18] and was performed in our institute with the following modifications. In brief: Classical Seldinger catheterization with an end-hole angiographic catheter was used. Arteriography of the celiac trunk or the superior mesenteric artery was obtained to visualize the arterial vascularization of the liver. The same catheter was used for both drug injection and embolization. Selective injection was performed unless technical difficulties prevented selective catheterization. If the hepatic artery was occluded, an attempt was made to catheterize extrahepatic collaterals supplying the liver such as the inferior diaphragmatic, gastro-duodenal and left gastric arteries. The therapeutic emulsion contained Adriamycin and Lipiodol. The emulsion was injected into the hepatic artery distal to the gastroduodenal artery origin. Gelatin sponge particles, 1-2 mm in diameter, were then utilized to embolize the feeding vessels until a markedly reduced flow was observed. Particle size and arterial slow-down intensity) as evaluated fluoroscopically) were adapted to the status of the hepatic portal perfusion, being less aggressive (larger particles and lesser degree of arterial slowdown) in cases of poor hepatic portal perfusion. Patients received 1.5 L/d of intravenous fluid from 24 h before to 48 h after treatment. Cefamezin (1 g) and Dexamethasone (10-20 mg) was given 1 h before the procedure. Patients underwent repeated TACE procedures according to tumor viability and clinical condition as assessed by a multi-disciplinary team.

### *Statistical analysis*

Mean and SD for continuous variables were used as indices of centrality and dispersion of the distribution. For non-normally distributed values it was necessary to use a non-parametric methods, The Wilcoxon rank-sum (Mann-Whitney) test, was used for continuous variables, to test the comparisons between the AgI categories of liver function parameters. The Cox proportional hazards model was applied to evaluate the predictive factors as categories of AgI score associated with overall survival. The results were presented as HR with 95%CI. Unconditional multiple logistic regression model was used to evaluate the Odds-Ratio of the AgI score ($\geqslant$ 4) on the dichotomized Gamma Glutamyl-Transpeptidase (GGTP). All variables were included together in the model. The results were presented as OR with 95%CI. In all models, Cox regression and Logistic regression, the HR and the OR respectively, represent the risk for one-unit variation of the predictor variable considered as dummy variables. Patient survival between the two categories of AgI score was estimated with the Kaplan-Meier method and comparison of survival was made with the Breslow (generalized Wilcoxon) test. The log rank test was used, due to the small proportion of patients who died early. When testing the hypothesis of significant association, *P*-value was < 0.05, two tailed

**Table 1 Multiple logistic regression of aggressiveness index (score = 4/score > 4) on liver function parameter**

| All models in total cohort | OR | se(OR) | *P* value | 95%CI |
|---|---|---|---|---|
| Variables included together in the model | | | | |
| Total Bilirubin (mg/dL) | 2.044 | 0.875 | 0.095 | 0.883 to 4.729 |
| ALKP (IU/mL) | 1.013 | 0.006 | 0.046 | 1.000 to 1.025 |
| GGTP (IU/mL) | 0.995 | 0.002 | 0.045 | 0.990 to 0.999 |
| AST (IU/L) | 1.002 | 0.003 | 0.442 | 0.996 to 1.009 |
| Albumin (g/dL) | 3.197 | 1.74 | 0.033 | 0.101 to 9.288 |
| Platelets (× $10^9$/L) | 1.014 | 0.008 | 0.056 | 1.000 to 1.029 |
| WBC (× $10^9$/L) | 0.811 | 0.121 | 0.158 | 0.606 to 1.085 |
| Lymphocyte (× $10^9$/L) | 1.311 | 0.354 | 0.315 | 0.773 to 2.227 |
| Final model from stepwise method in backward | | | | |
| ALKP (IU/mL) | 1.014 | 0.006 | 0.017 | 1.003 to 1.026 |
| GGTP (IU/mL) | 0.996 | 0.002 | 0.053 | 0.991 to 1.000 |
| Albumin (g/dL) | 2.562 | 1.082 | 0.026 | 1.120 to 5.863 |

Aggressiveness index (sum of scores): MTD (in tertiles): MTD < 4.5; 4.5 ⩽ MTD ⩽ 9.6; MTD > 9.6; scores 1, 2, 3 respectively; AFP (cut-off): AFP < 100; 100 ⩽ AFP ⩽ 1000; AFP > 1000; scores 1, 2, 3 respectively; PVT (no/yes): PVT (no); PVT (yes); scores 1, 3 respectively; nodules (number): Nodules ⩽ 3; nodules > 3; scores 1, 3 respectively. AFP: Alpha-fetoprotein; MTD: Maximum tumor diameter; PVT: Portal vein thrombosis; ALKP: Alkaline phosphatase; GGTP: Gamma glutamyl transpeptidae; AST: Aspartate aminotransaminase; Hb: Haemoglobin; Plt: Platelet count; WBC: White blood cell.

**Table 2 Characteristics of patients in the total cohort**

| Parameter[1] | Value |
|---|---|
| Age (yr) | 64.24 ± 10.35 |
| Sex (M) (%) | 124 (74.25) |
| Cirrhosis (yes) (%) | 134 (80.24) |
| AFP (ng/dL) | 1769.49 ± 7297.65 |
| AFP (median, range) | 53.80 (1-66000) |
| AFP > 100 (%) | 54 (40.60) |
| Number nodules | 1.95 ± 1.39 |
| MTD (cm) | 4.45 ± 2.64 |
| PVT (yes) (%) | 24 (14.37) |
| Aggressiveness index score (%) | |
| Score > 4 | 75 (63.56) |
| Total bilirubin (mg/dL) | 1.23 ± 0.80 |
| ALKP (IU/mL) | 133.29 ± 74.74 |
| GGTP (IU/mL) | 152.26 ± 152.80 |
| AST (IU/L) | 104.68 ± 110.73 |
| ALT (IU/L) | 77.85 ± 88.25 |
| Albumin (g/dL) | 3.63 ± 1.93 |
| Platelet count (× $10^9$/L) | 113.78 ± 82.70 |
| WBC (× $10^9$/L) | 5.46 ± 2.59 |
| Lymphocyte (× $10^9$/L) | 1.41 ± 0.98 |
| Survival time (median, range) | 38 (3-175) |

[1]All values: mean ± SD for continuous variables; Aggressiveness Index (sum of scores): MTD (in tertiles): MTD < 4.5; 4.5 ⩽ MTD ⩽ 9.6; MTD > 9.6; scores 1, 2, 3 respectively; AFP (cut-off): AFP < 100; 100 ⩽ AFP ⩽ 1000; AFP > 1000; scores 1, 2, 3 respectively; PVT (no/yes): PVT (no); PVT (yes); scores 1, 3 respectively; Nodules (number): Nodules ⩽ 3; Nodules > 3; scores 1, 3 respectively. AFP: Alpha-fetoprotein; MTD: Maximum tumor diameter; PVT: Portal vein thrombosis; ALKP: Alkaline phosphatase; GGTP: Gamma glutamyl transpeptidae; AST: Aspartate aminotransaminase; ALT: Alanina amminotransferasi; Hb: Haemoglobin; WBC: White blood cell.

for all analyses. Statistical analysis was performed with State Corp 2007 State Statistical Software: release 10. College Station, TX: StataCorp LP.

## RESULTS

### *Patients' characteristics*

A total of 167 patients were included in this study. Six patients were omitted because of missing data, leaving 161 patients in the final analysis. Sixty seven patients were under surveillance for their underlying liver disease. The median age was 64.24 ± 10.35 years, the majority were males (*n* = 124, 74.25%) and 80.24% had cirrhosis with complications, including ascites (*n* = 40), varices (*n* = 67), encephalopathy (*n* = 20), and abdominal pain (*n* = 34) at diagnosis. Etiologies of the underlying liver disease included: HCV (*n* = 91); HBV (*n* = 35); NASH (*n* = 17); cryptogenic cirrhosis (*n* = 17); HCV and HBV (*n* = 3); ASH and HCV (*n* = 2); Autoimmune hepatitis (*n* = 1), Alcoholic steatohepatitis (*n* = 1). The mean AFP levels were 53.8 (range: 1-66000). Mean number of tumor nodules was 1.95 + 1.39 and the MTD was 4.45 ± 2.65 cm. Twenty four patients (14.37%) had PVT. Mean serum ALKP, GGTP, bilirubin and albumin levels were 104.68 ± 110.73 IU, 152.26 ± 152.8 IU, 1.23 ± 0.80 (mg/dL) and 3.63 ± 1.93 (g/dL) respectively (Table 2).

### *Survival analysis and AgI*

Median survival from the time of HCC diagnosis to death or transplantation was 38 mo (range 3-175 mo) (Table 2). Seventy five (63.5%) patients had a score of > 4 on the AgI (Table 2). The AgI was correlated with survival. The 3-year survival probability for AgI of > 4 *vs* < 4 was 42.4% *vs* 61.8%; *P* < 0.0863, from the time of diagnosis by Kaplan-Meier plot (Figure 1). Moreover, according to the univariate Cox proportional hazard model for mortality with AgI score of > 4, there was a HR of 2.18 (95%CI: 1.108-4.310, *P* < 0.024) (Table 3).

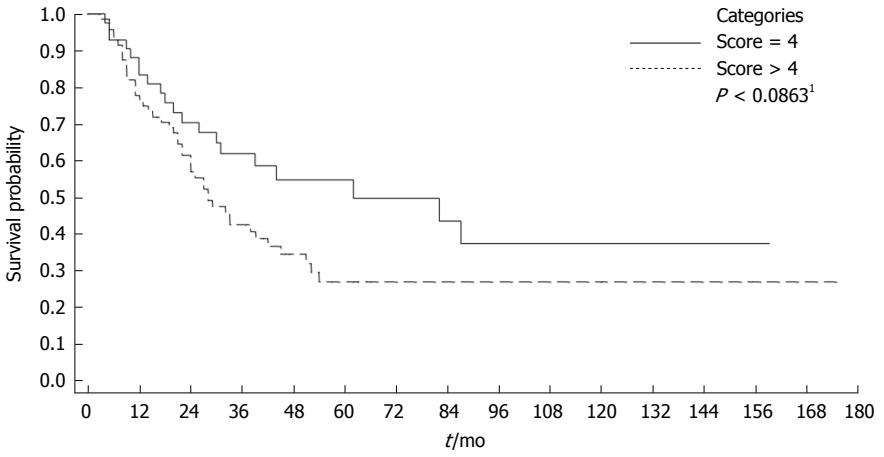
### *Correlation of AgI with other parameters*

A univariate multiple logistic regression of AgI with 8 laboratory parameters was obtained and two baseline laboratory parameters were found to independently correlate with the AgI score (Table 1). Albumin's single unit change was associated with an OR of 3.19

**Table 3 Cox proportional hazard model for death on aggressiveness index score and liver function parameters**

| All models in total cohort | HR | se(HR) | *P* value | 95%CI |
|---|---|---|---|---|
| Variables included together in the model | | | | |
| Aggressiveness Index | | | | |
| Score = 4 [Ref. category] | 1 | - | - | - |
| Score > 4 | 2.185 | 0.757 | 0.024 | 1.108 to 4.310 |
| Total bilirubin (mg/dL) | 0.985 | 0.154 | 0.925 | 0.725 to 1.339 |
| ALKP (IU/mL) | 0.999 | 0.003 | 0.793 | 0.993 to 1.005 |
| GGTP (IU/mL) | 1.003 | 0.001 | 0.016 | 1.001 to 1.006 |
| AST (IU/L) | 0.999 | 0.002 | 0.511 | 0.995 to 1.003 |
| Albumin (g/dL) | 0.793 | 0.250 | 0.462 | 0.427 to 1.472 |
| Platelets ($\times 10^9$/L) | 1.002 | 0.002 | 0.161 | 0.999 to 1.006 |
| Final model from stepwise method in backward | | | | |
| Aggressiveness Index | | | | |
| Score = 4 [Ref. category] | 1 | - | - | - |
| Score > 4 | 2.263 | 0.748 | 0.013 | 1.184 to 4.327 |
| GGTP (IU/mL) | 1.003 | 0.001 | 0.001 | 1.001 to 1.004 |

Aggressiveness index (sum of scores): MTD (in tertiles): MTD < 4.5; 4.5 ⩽ MTD ⩽ 9.6; MTD > 9.6; scores 1, 2, 3 respectively; AFP (cut-off): AFP < 100; 100 ⩽ AFP ⩽ 1000; AFP > 1000; scores 1, 2, 3 respectively; PVT (no/yes): PVT (no); PVT (yes); scores 1, 3 respectively; nodules (number): Nodules ⩽ 3; nodules > 3; scores 1, 3 respectively. AFP: Alpha-fetoprotein; MTD: Maximum tumor diameter; PVT: Portal vein thrombosis; ALKP: Alkaline phosphatase; GGTP: gamma glutamyl transpeptidae; AST: Aspartate aminotransaminase; Hb: Haemoglobin; Plt: Platelet count.




| | Survival probability (%) | | |
|---|---|---|---|
| Categories | 1 yr | 2 yr | 3 yr |
| Score = 4 | 83.3 | 70.4 | 61.8 |
| Score > 4 | 76.3 | 56.9 | 42.4 |

**Figure 1 Kaplan-Meier survival plots between categories of aggressiveness Index, in total cohort.** Aggressiveness index as sum of scores, MTD (in tertiles): MTD < 4.5; 4.5 ⩽ MTD ⩽ 9.6; MTD > 9.6; scores 1, 2, 3 respectively; AFP (cut-off): AFP < 100; 100 ⩽ AFP ⩽ 1000; AFP > 1000 ng/mL; scores 1, 2, 3 respectively; PVT (no/yes): PVT (no); PVT (yes); scores 1, 3 respectively; Tumor nodules (number): Nodules ⩽ 3; nodules > 3; scores 1, 3 respectively. [1]Wilcoxon (breslow) test. MTD: Maximum tumor diameter; AFP: Alpha-fetoprotein; PVT: Portal vein thrombosis.

(95%CI: 0.101-9.299), followed by ALKP with an OR of 1.01 (95%CI: 1.000-1.025) (Table 1). These two parameters were then assessed in a multivariate multiple logistic regression to a final model which showed the following correlation: Albumin with an OR of 2.56 (95%CI: 1.120-5.863) followed by ALKP with an OR of 1.01 (95%CI: 1.003-1.026) (Table 1).

A univariate Cox proportional hazard model for mortality with AgI score and liver function parameters was performed. We found that only GGTP levels and the AgI were independently associated with survival of the HCC patients following TACE (Table 3). We used our cohort to validate the previously described AgI. An AgI with the score > 4 had HR for mortality of 2.18 (95%CI: 1.108-4.310, $P < 0.024$). GGTP's single unit change had an HR for mortality of 1.003 (95%CI: 1.001-1.006, $P < 0.016$). We considered them in the final multivariate model with the total cohort. An AgI with the score > 4 had an HR for mortality of 2.26 (95%CI: 1.184-4.327, $P < 0.016$). GGTP had an HR of 1.003 (95%CI: 1.001-1.004, $P < 0.001$) (Table 3).

### *Comparison of GGTP level groups*

We then compared the HCC patients (Table 4) dichotomized by GGTP levels of 100IU/L (< 100/> 100) based on a previous finding that there is a marked

**Table 4 Comparisons in hepatocellular carcinoma patients among dichotomization of gamma glutamyl transpeptidase (⩽ 100/> 100 IU/L), in the total cohort**

| Parameter[1] | GGTP (IU/L) | | $P^2$ value |
|---|---|---|---|
| | ⩽ 100 ($n$ = 81) (50.31%) | > 100 ($n$ = 80) (49.69%) | |
| Total bilirubin (mg/dL) | 1.25 ± 0.75 | 1.19 ± 0.87 | 0.17 |
| ALKP (IU/mL) | 104.55 ± 42.06 | 160.92 ± 89.79 | < 0.0001 |
| GGTP (IU/mL) | 55.44 ± 24.46 | 250.29 ± 165.35 | < 0.0001 |
| AST (IU/L) | 93.61 ± 79.10 | 115.45 ± 137.27 | 0.06 |
| Albumin (g/dL) | 3.70 ± 2.68 | 3.56 ± 0.57 | 0.13 |
| Platelets (× $10^9$/L) | 100.14 ± 61.45 | 129.60 ± 100.06 | 0.02 |
| Aggressiveness index (%) | | | |
| Score > 4 | 33 (56.90) | 42 (75.00) | 0.04[3] |
| Survival at time (%) | | | |
| 1 yr | 68 (87.18) | 54 (70.13) | 0.01[3] |
| 2 yr | 52 (66.67) | 37 (48.05) | 0.02[3] |
| 3 yr | 39 (50.00) | 25 (32.47) | 0.03[3] |

[1]All values: mean ± SD; [2]Wilcoxon rank-sum (Mann-Whitney) test; [3]Test Z for proportions. Aggressiveness index (sum of scores): MTD (in tertiles): MTD < 4.5; 4.5 ⩽ MTD ⩽ 9.6; MTD > 9.6; scores 1, 2, 3 respectively; AFP (cut-off): AFP < 100; 100 ⩽ AFP ⩽ 1000; AFP > 1000; scores 1, 2, 3 respectively; PVT (no/yes): PVT(no); PVT(yes); scores 1, 3 respectively; nodules (number): Nodules ⩽ 3; nodules > 3; scores 1, 3 respectively. AFP: Alpha-fetoprotein; MTD: Maximum tumor diameter; PVT: Portal vein thrombosis; ALKP: Alkaline phosphatase; GGTP: Gamma glutamyl transpeptidae; AST: Aspartate aminotransaminase; Hb: Haemoglobin; Plt: Platelet count.

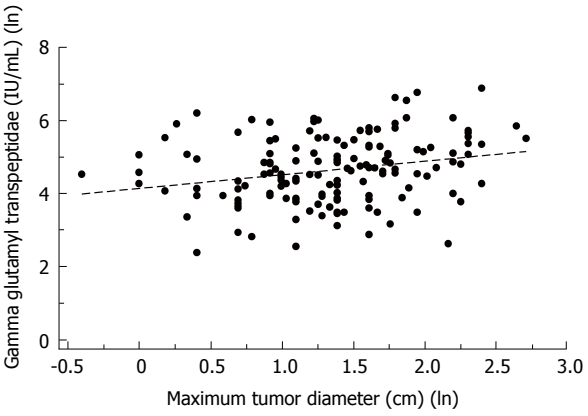



**Figure 2 Scatterplots between maximum tumor diameter (cm) and gamma glutamyl transpeptidae (IU/mL) (Spearman's rho = 0.2604, $P$ = 0.0012), together with linear regression line of gamma glutamyl transpeptidae on maximum tumor diameter, in total cohort. All transformed into natural logarithm.** ln, natural logarithm; Fitted values (-------).

difference in survival of patients with HCC between these values[19]. The GGT*P* < 100 group had 81 patients and the GGTP > 100 had 80 patients. The GGTP > 100 group had higher liver enzyme levels: ALKP = 160.92 + 89.79 *vs* 104.55 + 42.06; respectively ($P$ < 0.0001) (Table 4). The 1 and 3 year survival was 17% higher in the GGTP (< or =) 100 group (87.18% *vs* 70.1% and 50% *vs* 32.47%, $P$ = 0.01 and $P$ = 0.03, respectively) (Table 4). We also assessed correlation between laboratory parameters and MTD. Gamma-glutamyl-transpeptidase levels (IU/mL) and the Maximum Tumor Diameter (cm) showed a low positive correlation with a Spearman's coefficient of $r$ = 0.2604 and a $P$-value of 0.0012 with a linear regression line (Figure 2).

## DISCUSSION

Tumor factors and liver function parameters were shown to have prognostic value in predicting survival of HCC patients. Our goal was to validate the usefulness of the recently-described HCC AgI, in a novel HCC cohort and to assess its usefulness in patients that underwent TACE while trying to identify additional laboratory serum parameters to improve its accuracy. We excluded patients with fibrolamellar HCC, mixed cholangio-hepatocellular carcinoma and sarcomatous type HCC, because of their rarity and their variant clinical course which may be different then "regular" HCC.

The AgI was recently reported and includes the following tumor parameters: maximum tumor diameter, number of tumor nodules and presence of PVT and plasma AFP levels.

Our cohort included patients with multiple underlying liver diseases and can be widely generalized, in contrast to the original cohort, where the underlying etiology of the liver disease was not specified.

HCC tumor parameters were previously shown to have prognostic value and were used in various staging systems[20]. In a 2011 paper by Hu *et al*[7], analyzing data from 362 patients undergoing TACE, all 4 of the AgI parameters were found to be independently significant predictors of patient's survival. Maximal tumor size (HR = 1.66, $P$ < *0*.002), Portal vein invasion (HR = 2.39, $P$ < *0*.001), Tumor nodule number (HR = 1.92, $P$ < *0*.001), and AFP value (HR = 1.54, $P$ < *0*. 003). Portal vein invasion was associated with a marked decrease in patients' survival, while the other parameters had only a modest effect on survival. However, combining them in the AgI increased their predictive power considerably. Furthermore, in their original paper the authors used single categories for each parameter, whereas the AgI divides each parameter into tertiles refining the scoring. We similarly show that combining these parameters in our specific population of patients that underwent TACE increases

their predictive power.

Our patients had smaller tumors and longer survival time for both of the < 4 and > 4 score groups compared to previously reported groups[21-23]. The fact that most of the patients had an underlying liver disease and 40% were under a strict surveillance program made it possible to detect HCC at an early stage.

We found a significant increase in HR for death in patients with an AgI > 4, thus expanding the original observation to our patient population (Table 3).

Our second goal was to examine possible correlation between the AgI and other laboratory parameters of liver function (Table 1). We focused on 8 parameters that were previously shown to be associated with prognosis in HCC patients. Included were albumin and bilirubin which are part of the CPT score. Also included were markers of liver damage such as AST, ALK and GGTP[15]. Finally we looked at the hematologic parameters; platelets that were previously shown to be associated with tumor aggressiveness[16] and WBC and Lymphocytes, that were considered because Neutrophils to Lymphocytes ratio predicted overall survival in HBV-related HCC patients after TACE[13].

Other parameters that may better predict liver function or tumor aggressiveness such as Indocyanine Green (ICG) clearance[24] and Des-Gamma carboxyprothrombin (DCP) were excluded because they were not routinely utilized in our center and are not in wide clinical use[25,26].

In contrast to previous reports[10,11,27], and although albumin was correlated to AgI, we were unable to show that albumin is an independent predictor of survival in our cohort. We attribute this discrepancy to the fact that in a large portion of our cohort the tumor was discovered early when tumor parameters were favorable and liver function relatively preserved. Therefore, most of the patients ($n$ = 110) had Albumin levels within the normal range.

Similar to previous reports[15,16] we also found elevated GGTP, to be an independent risk factor for mortality (HR = 1.003; 95%CI: 1.001-1.004; $P$ < 0.001). In earlier publications, elevated levels of GGTP were found to be a poor prognostic factor after liver transplantation and before liver resection as they were associated with advanced tumor stage and aggressive tumor behaviors[28,29]. This factor can now be used as a predictor of prognosis in the population of TACE treated HCC patients.

We divided GGTP into two groups with a cutoff of 100IU/L (< 100/> 100). The group with the higher GGTP (> 100) had higher AgI score (52.5% compared with 40.7%) and lower survival at 1 and 3 years. (87.18% *vs* 70.1% and 50% *vs* 32.47%, $P$ = 0.01, 0.03 respectively). In a recently published paper by Barman *et al*[30], focusing on patients undergoing TACE, it was noted that survival was higher among those patients with well-preserved liver synthetic function.

We were unable to show that other laboratory parameters associated with CPT (Bilirubin and INR-data not shown) were independently associated with prognosis. Other studies assessing these factors, show conflicting results. A previously published study found that elevated bilirubin (HR = 4.2; 95%CI: 2.2-7.9; $P$ < 0.001) was a significant independent risk factor for mortality in 84 consecutive patients with HCC treated with TACE as first-line or second-line treatment that were enrolled between 2004 to 2009[31]. In contrast, a study of 109 patients who underwent TACE from 2006 to 2012 in a veterans hospital in the United States, did not show any single component of Child-Pugh score to be a predictor of survival[30]. The authors explain their results stating that their population was mostly males with HCV and dissimilar to previously published studies in which the population was mostly Asian with HBV and preserved liver function. These discrepancies suggest that different factors may predict tumor aggressiveness in different patient populations.

We concluded that the AgI is a validated tool to predict overall survival in unresectable HCC patients undergoing the TACE procedure. We further suggest that it can be combined with elevated GGTP levels, elevated levels of ALKP and decreased levels of albumin to improve its prognostic yield in this patient population.

## ARTICLE HIGHLIGHTS

### *Research background*

Hepatocellular carcinoma (HCC) is a common and deadly cancer. Transterial chemoembolization (TACE) is the treatment of choice for non-operable, intermediate stage HCC.

### *Research motivation*

There is a need to identify prognostic indices in HCC patients undergoing TACE. An "HCC aggressiveness index (AgI)" incorporates 4 tumor-related parameters: maximum tumor diameter (MTD), number of tumor nodules, portal vein thrombosis (PVT) and serum alpha fetoprotein (AFP) levels. This score predicts survival in HCC patients.

### *Research objective*

To identify novel biomarkers to predict survival following TACE and combine them with the AgI.

### *Research methods*

We retrospectively analyzed data from 167 patients with HCC that underwent TACE at Tel-Aviv Medical center from 2000 to 2015. Baseline tumor parameters including: maximum tumor diameter, number of tumor nodules and presence of PVT; labs including: blood count; routine liver function tests and plasma AFP levels; demographics and overall survival information were all collected. The Cox proportional hazards model was applied to identify the correlation of AgI with overall survival and analyze laboratory factors' associated with the AgI.

### *Research results*

The AgI was correlated with survival. The 3-year survival probability for AgI of > 4 *vs* < 4 was 42.4% *vs* 61.8%; $P$ < 0.0863, from the time of diagnosis by Kaplan-Meier plot. Moreover, According to the univariate Cox proportional hazard model for mortality with AgI score of > 4, there was a HR of 2.18 (95%CI: 1.108-4.310, $P$ < 0.024). We found that only GGTP levels and the AgI were independently associated with survival of the HCC patients following TACE.

### *Research conclusions*

AgI was validated as a useful predictor of survival in HCC patients undergoing TACE. Combining the AgI with liver function parameters may improve its prognostic yield in this patient population.

### *Research prospective*

This novel score can be used to assess prognosis in HCC undergoing TACE.

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*World J Gastroenterol* 2018 April 21; 24(15): 1650-1657

DOI: 10.3748/wjg.v24.i15.1650

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Observational Study

# Development and predictive validity of the cirrhosis-associated ascites symptom scale: A cohort study of 103 patients

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Author contributions: Gluud LL designed the study and drafted the protocol; Gluud LL, Jensen AS and Dahl EK collected questionnaires and conducted interviews for the pilot-testing of the cirrhosis-associated ascites symptom (CAS); Riedel AN, Jensen AS, Dahl EK, Gluud LL, Aamann L, Israelsen M and Kimer N collected questionnaires for the validation of CAS; Riedel AN and Gluud LL performed the analyses; Kimer N, Riedel AN and Gluud LL drafted the first version of the manuscript. All authors critically revised and approved of the final version of the manuscript.

Institutional review board statement: The study was approved by the Danish Data protection Agency, journal no: 04054, ID: AHH-2015-075.

Informed consent statement: All participants signed an informed consent.

Conflict-of-interest statement: The authors declare no conflicts of interest.

Data sharing statement: No additional data are available.



Manuscript source: Unsolicited manuscript

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Received: November 28, 2017
Peer-review started: November 29, 2017
First decision: December 13, 2017
Revised: December 20, 2017
Accepted: January 18, 2018
Article in press: January 18, 2018
Published online: April 21, 2018

## Abstract

***AIM***

To develop a scale of domains associated with the health-related quality-of-life (HRQOL) in patients with cirrhosis-related ascites.

***METHODS***

We initially undertook literature searches and a qualitative study in order to design a cirrhosis-associated ascites symptom (CAS) scale describing symptoms with a potential detrimental impact on health related quality of life (HRQL) (the higher the score, the worse the symptoms). Discriminatory validity was assessed in a validation cohort including cirrhotic patients with (1) tense/severe; (2) moderate/mild; or (3) no ascites (controls). Patients also completed chronic liver disease questionnaire (CLDQ) and the EuroQoL 5-Dimensions 5-Level (EQ-5D-5L) questionnaire evaluating HRQL. The relation between scale scores was analysed using Spearman correlations.

***RESULTS***

The final CAS scale included 14 items. The equivalent reliability was high (Chronbach's alpha 0.88). The validation cohort included 103 patients (72% men, mean age 62.4 years). The mean scores for each question in the CAS scale were higher for patients with severe/tense ascites than for mild/moderate ascites and controls. Compared with controls (mean = 9.9 points), the total CAS scale score was higher for severe/tense ascites (mean = 23.8 points) as well as moderate/mild ascites (mean = 18.6 points) ($P < 0.001$ both groups). We found a strong correlation between the total CAS and CLDQ score (rho = 0.82, $P < 0.001$) and a moderate correlation between the CAS and the EQ-5D-5L score (0.67, $P < 0.001$).

***CONCLUSION***

The CAS is a valid tool, which reflects HRQOL in patients with ascites.

**Key words:** Health-related quality-of-life; Cirrhosis; Symptom burden; Symptom assessment



**Core tip:** This paper presents a newly generated cirrhosis-associated ascites symptom scale consisting of 14 items. The questionnaire addresses relevant questions of symptom burden of cirrhosis-associated ascites, takes only five minutes to complete and correlates strongly with chronic liver disease questionnaire score in patients with cirrhosis and ascites.

Riedel AN, Kimer N, Jensen AS, Dahl EK, Israelsen M, Aamann L, Gluud LL. Development and predictive validity of the cirrhosis-associated ascites symptom scale: A cohort study of 103 patients. *World J Gastroenterol* 2018; 24(15): 1650-1657 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i15/1650.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i15.1650

## INTRODUCTION

Ascites is a serious complication to cirrhosis[1,2]. Without liver transplantation, only about 55% of patients with cirrhosis and ascites are alive, five years after the initial diagnosis. In addition, the severity of symptoms associated with ascites may have a detrimental impact on health-related quality of life (HRQL). Five previous studies have evaluated the HRQL in patients with cirrhosis using generic questionnaires including the Medical Outcome Study Short Form 36 (SF-36), the Nottingham Health Profile and the Sickness Impact Profile[3-7]. All studies found that cirrhosis is associated with a detrimental effect on HRQL. None of the studies were designed to evaluate the association between ascites severity and the quality of life. However, one study found that disease progression, including development of ascites, is associated with an impaired HRQL[7]. Disease-specific questionnaires developed for the assessment of HRQL in patients with liver disease and cirrhosis have reached similar conclusions[8,9]. The questionnaires include aspects that are of particular relevance to patients with cirrhosis, such as concerns about complications and liver transplantation. The 29 item chronic liver disease questionnaire (CLDQ) was used to evaluate HRQL in 204 patients representing all stages of various liver diseases[10]. The scale correlates with the severity of the underlying liver disease and is also associated with active medical and psychiatric comorbidity. Liver failure, development of minimal hepatic encephalopathy, malnutrition, abdominal pain, fatigue, and anxiety may also affect HRQL[11,12].

None of previous studies specifically evaluated the association between the severity of ascites related symptoms and HRQL. However, it is likely that the severity of symptoms is important.

In clinical trials of medical interventions, HRQL as well as symptom management are important parameters in the assessment of intervention benefits and harms[4,5,13,14]. A study including 212 outpatients with cirrhosis found that the management of cirrhosis-related complications is associated with improved HRQOL assessed using the Medical Outcomes Study Form, SF-36 and CLDQ questionnaires. The study found that ascites as well as a decrease in haemoglobin and previous hepatic encephalopathy predicted the HRQOL[15].

We therefore developed and evaluated the predictive ability of a scale specifically made to assess symptoms related to cirhosis-associated ascites. We subsequently undertook a multicenter cohort study to evaluate the association between our scale and a disease specific scale (CLDQ) as well as a generic HRQL questionnaire (EQ-5D-5L).

## MATERIALS AND METHODS

The present study was conducted as a multi-center study, with participation of three university hospitals in Denmark: Copenhagen University Hospital Hvidovre, Odense University Hospital and Aarhus University Hospital. The study was approved by the Danish Data protection Agency, journal no: 04054, ID: AHH-2015-075.

The development of the cirrhosis-associated ascites symptom (CAS) scale included a literature review and a

**Table 1 Cirrhosis-associated ascites symptom scale in Danish**

| Please answer the following questions with a cross or tick with regards to how you have felt in the past four weeks. | | | | |
|---|---|---|---|---|
| | **Nej** *No* | **Sjældent** *Rarely* | **Indimellem** *Sometimes* | **Ofte** *Often* |
| 1 Har du ondt i maven? *Do you have stomach ache?* | | | | |
| 2 Har du ømhed i maven? *Does your stomach feel sore?* | | | | |
| 3 Har du kvalme? *Do you feel nauseous?* | | | | |
| 4 Har du madlede? *Do you feel an aversion for food?* | | | | |
| 5 Har du nedsat appetit? *Do you experience a loss of appetite?* | | | | |
| 6 Har du ændret afføring? *Have you experienced a change in your stool?* | | | | |
| 7 Har du følt dig utilpas? *Have you felt uncomfortable?* | | | | |
| 8 Er du træt? *Are you tired?* | | | | |
| 9 Har du åndenød? *Do you experience a shortness of breath?* | | | | |
| 10 Har du svært ved at trække vejret helt igennem? *Do you feel troubled when breathing deeply?* | | | | |
| 11 Har du svært ved at få tøj eller sko på? *Are you disabled when dressing or putting on shoes?* | | | | |
| 12 Har du svært ved at komme rundt derhjemme? *Do you experience difficulties in mobility at home?* | | | | |
| 13 Har du svært ved at gå? *Do you experience trouble walking?* | | | | |
| 14 Har du ondt i benene? *Do your legs hurt?* | | | | |

qualitative study to evaluate face and content validity as well as discriminatory ability.

We initially searched for previously validated scales in MEDLINE and EMBASE ('ascites' AND ('quality of life [Mesh]' OR 'life quality' OR 'health-related quality of life' OR 'health related quality of life' OR 'symptoms') AND ('cirrhosis' OR 'end-stage liver disease'). The searches were combined with manual searches of reference lists in potentially relevant articles and conference proceedings. None of our searches identified questionnaires evaluating symptoms associated with ascites.

We then proceeded with a qualitative study. Initially, the study group and ten independent experts identified and listed what they believed were the most important symptoms with an expected negative impact on HRQL (six hepatologists and four nurses with clinical experience in the management of patients with ascites from Gastro Unit, medical division, Copenhagen University Hospital Hvidovre, were interviewed). In our selection of symptoms (domains), we decided that they should occur frequently or be severe enough to have substantial impact on daily function in order to be considered for our scale.

After the selection of domains, we first conducted open interviews followed by structured interviews of ten patients with cirrhosis and moderate ($n$ = 4) or severe ascites ($n$ = 6). The interviews took place in a quiet room in the hospital ward. The selected domains were initially presented and patients were asked to rate them (Supplementary Table 1) according to the patient's perceived 'importance', on the extent to which the symptom had bothered them. Each patient was then asked to complete the preliminary questionnaire themselves and to answer a debriefing form with the questions: (1) How long did it take to complete the questionnaire? (2) Did you need assistance from others to fill out the questionnaire? (3) Did you find any items confusing or difficult to respond to (and if so, list the items)? (4) Did you find any items upsetting? (5) Were any important items missing? and (6) Do you have any additional comments?

We revised the questionnaire instrument based on the answers. One question, which patients found upsetting and two questions, which patients found to be irrelevant or unimportant were removed. Revisions were decided by consensus among investigators.

### *Validation of the CAS scale (known group comparison)*

After the development of the questionnaire, we prospectively enrolled a validation cohort (known group comparison) consisting of patients with cirrhosis of any aetiology and (1) severe/tense ascites; (2) mild/moderate ascites; and (3) no ascites as determined through a combination of clinical assessments and abdominal ultrasound. All gave their informed consent to participate. Subjects were attending out-patient clinics or were admitted to hospital ward with ascites as their

**Table 2 Patient characteristics according to degree of ascites *n* (%)**

| | Control group, no ascites (*n* = 31) | Mild/moderate (*n* = 27) | Tense (*n* = 45) | Total (*n* = 103) |
|---|---|---|---|---|
| Age: mean (SD) | 61.5 (8.4) | 62.3 (10.2) | 63.1 (11.3) | 62.4 (10.1) |
| Gender: Males | 23 (74) | 19 (70) | 32 (71) | 74 (72) |
| Etiology | | | | |
| Alcohol | 28 (90) | 22 (81) | 38 (86) | 88 (86) |
| Viral | 1 (3) | 2 (7) | 3 (7) | 6 (6) |
| Alcohol and viral | 1 (3) | 1 (4) | 3 (7) | 5 (5) |
| Other | 3 (10) | 4 (15) | 6 (13) | 13 (13) |
| MELD score: mean (SD) | 10.1 (3.3) | 14.8 (6.2) | 15.9 (6.3) | 13.8 (6.0) |
| Child PUGH: mean (SD) | 6.2 (1.8) | 8.1 (1.3) | 9.1 (1.4) | 7.9 (1.9) |
| Class A | 19 (61) | 4 (15) | - | 23 (22) |
| Class B | 7 (23) | 17 (63) | 27 (60) | 51 (50) |
| Class C | 2 (6) | 2 (7) | 12 (27) | 16 (16) |
| Laboratory values (SD) | | | | |
| Hemoglobin (mmol/L) | 8.4 (1.4) | 7.1 (1.2) | 6.4 (1.4) | 7.2 (1.6) |
| White blood cells ($\times 10^9$/L) | 6.9 (2.6) | 7.2 (3.9) | 7.3 (3.5) | 7.1 (3.3) |
| Platelets (mmol/L) | 145 (65) | 158 (95) | 160 (87) | 155 (83) |
| Albumin (g/L) | 35 (5.8) | 29 (5.9) | 30 (14) | 31 (10) |
| Cogulation factor Ⅱ, Ⅶ, X (INR) | 1.3 (0.3) | 1.4 (0.3) | 1.5 (0.5) | 1.4 (0.4) |
| Creatinine (μmol/L) | 74 (17) | 87 (29) | 94 (48) | 86 (37) |
| Sodium (mmol/L) | 138 (3.6) | 134 (5.9) | 132 (6.9) | 135 (6.3) |
| Potassium (mmol/L) | 4.1 (0.4) | 4.3 (0.5) | 4.2 (0.7) | 4.2 (0.6) |

There were no patients in Child Pugh Class A with tense ascites.

primary problem. Subjects with hepatic encephalopathy or other cognitive disability were excluded.

Subjects completed the following scales and questionnaires: (1) CAS scale; (2) Chronic Liver Disease Questionnaire[9]; and (3) EQ-5D-5L questionnaire[16,17].

### *Statistical analysis*

Statistics were calculated using STATA IC v14 R2 and GraphPad Prism v6. We used the Crohnbach's alpha to assess equivalent reliability. We defined *discriminant validity* as the ability of the questionnaire to discriminate between the severity of ascites. Discriminant validity was evaluated using Dunnett's test to adjust for multiple comparisons. The severe and mild ascites groups (assuming that this group has fewer and less severe symptoms than patients with severe ascites) were compared with the control group and *P*-values < 0.05 were considered significant. We originally planned to evaluate convergent validity with previously validated scales assessing symptoms or HRQOL associated with ascites. Since no previous scales were identified, we used the CLDQ and the EQ-5D-5L. We compared scale scores using Spearman correlation and included the (1) CAS scale; (2) CLDQ; (3) CLDQ subscale parameters; and (4) EQ-5D-5L. The CLDQ subscale parameters are defined domains consisting of specific questions in the CLDQ questionnaire. These domains include fatigue, emotional function, worry, activity, abdominal symptoms and systemic symptoms[9].

Correlations higher than 0.70 were considered strong and values below 0.40 were interpreted as poor.

## RESULTS

The final scale included 14 items (Table 1). Chronbach's alpha was 0.88 for the total score, which we considered as acceptable. The validation cohort included 103 patients.

Demographic data are stated in Table 2. Seventy-four were male (72%) and mean age was 62.4 years (range 38-85). The proportion of patients with Child Pugh A was 24%, Child Pugh B: 58% and Child Pugh C: 19%. Forty-four percent had severe/tense ascites and 27% had mild/moderate ascites. A control group of 31 patients (30%) had no ascites.

### *Discriminant validity*

The mean scores for each question in the CAS scale suggested that symptoms were worse for patients with severe/tense ascites than for controls (Figure 1).

Symptoms also appeared worse for patients with mild/moderate ascites. Accordingly, the CAS scale score found that patients with severe/tense ascites or moderate/mild ascites had significantly worse scores compared with controls ($P < 0.001$ and $P < 0.001$; Figure 2).

As expected, patients with severe/tense ascites had the worst symptoms scores. Based on the CLDQ questionnaire, tense/severe ascites had a detrimental impact on HRQOL compared with controls ($P < 0.001$) as did moderate/mild ascites ($P = 0.002$). The EQ-5D-5L also found a lower HRQOL in patients with severe/tense ascites ($P = 0.002$) or moderate/mild ascites ($P = 0.038$).

### *Convergent validity*

We found a strong correlation between the CAS and the CLDQ total score (Spearman's rho = 0.82, $P < 0.001$) as well as the CLDQ subscores; fatigue (0.78, $P < 0.001$), activity (0.81, $P < 0.001$) and systemic

**Table 3 Correlation between cirrhosis-associated ascites symptom score, EuroQoL 5-Dimensions 5-Level scale questionnaire and chronic liver disease questionnaire total score and subscores**

| | Rho | *P* value |
|---|---|---|
| Total EQ-5D-5L | -0.67 | < 0.001 |
| Total CLDQ score | -0.82[1] | < 0.001 |
| Fatigue | -0.78[1] | < 0.001 |
| Emotional function | -0.55 | < 0.001 |
| Worry | -0.47 | < 0.001 |
| Abdominal symptoms | -0.66 | < 0.001 |
| Activity | -0.81[1] | < 0.001 |
| Systemic symptoms | -0.77[1] | < 0.001 |

[1]Strong correlations. CLDQ: Chronic liver disease questionnaire; EQ-5D-5L: EuroQoL 5-Dimensions 5-Level scale questionnaire.

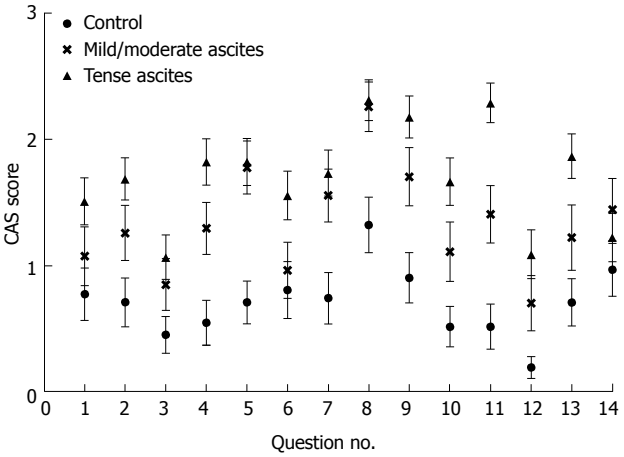


**Figure 1 Mean cirrhosis-associated ascites symptom scores by question number and ascites severity.**

symptoms (0.77, $P < 0.001$) (Table 3). The CAS correlation with the generic scale (EQ-5D-5L) was moderate (0.67, $P < 0.001$).

## DISCUSSION

We found that the developed CAS scale is effective in discriminating between various severities of ascites. It takes five minutes to perform and is easily implemented in daily practice. Moreover the CAS scale correlates strongly with the well-developed liver disease-specific HRQL instrument, CLDQ[9]. This suggests that our symptom assessment scale reflects the perceived HRQL of patients with cirrhosis and ascites. The CLDQ subparameters: fatigue, activity and systemic symptoms were likewise strongly correlated with the CAS scale suggesting that these domains are influential symptoms or functional limitations for patient with ascites. Surprisingly, the subparameter abdominal symptoms was not associated with the CAS scale. This may be due to use of words or inaccuracy of the questions, and will need further investigations for clarification. The total CLDQ was better correlated with CAS than each subparameter. This may be due to the impact of low quality of life affecting all aspects of life, allowing a total low score higher statistical significance. The CAS versus EQ-5D-5L correlation was moderate. The EQ-5D-5L is a generic scale which aims at examining non-disease specific HRQL[16,18,19], and specific symptoms related to cirrhosis and ascites may be undetected by this questionnaire. On the contrary, the CLDQ is created for patients with chronic liver disease, and it includes questions concerning ascites related symptoms and gains a stronger correlation with the CAS score.

To our knowledge, no previous study has developed and validated a questionnaire specifically aimed at evaluating HRQL in patients with ascites. However, previous literature describes correlations between disease severity in chronic liver disease and HRQL[7,12,20]. One study found that ascites had significant effects on self-related disease progression in cirrhosis[7]. Another showed that ascites was not a predictor for HRQL[12] and two studies found that management of ascites may improve HRQL[15,21]. In a study evaluating the impact of the Medical Outcomes Study Short Form (SF-36) on HRQL in 523 patients with cirrhosis and ascites, a strong association between the physical component score (PCS) and leg edema, history of previous hepatic encephalopathy, severe ascites and low serum sodium levels were found[22]. The PCS includes questions on physical role, physical functioning, and body pain and general health. Also, a lower mental component score in the SF-36 was associated with low levels of serum sodium and treatment with lactulose. Hence, several factors influence HRQL when measured by SF36 and the impact of ascites was not clearly established by this study.

The CAS score comprises only 14 questions with high compliance to answering, takes only five minutes to complete and is readily implemented in daily clinic. The present study supports the results implying correlation between ascites severity and HRQL assuming that the CAS scale is valid and reliable. Progression in liver disease (*e.g.,* measured by Child Pugh score which includes presence of ascites) is associated with ascites severity grade. This argues that our results support previous literature finding a correlation between liver disease severity and HRQL.

Our study has some considerable weaknesses. The relatively small sample size is one of the main limitations. Moreover, the majority of patients had cirrhosis due to alcohol. Dependency of alcohol, concomitant diseases related to alcohol use or cirrhosis that may impact physical health was not assessed in our cohort. Malnutrition is a serious complication to cirrhosis with impact on HRQL[23]. Also, impact of socioeconomic factors may influence quality of life substantially[24,25]. Future studies may address the synergy between various factors affecting quality of life in cirrhosis and ascites. The CAS scale is a specific scale evaluating the impact of symptoms related to ascites. When assessing overall quality of life on chronic liver disease or the impact of

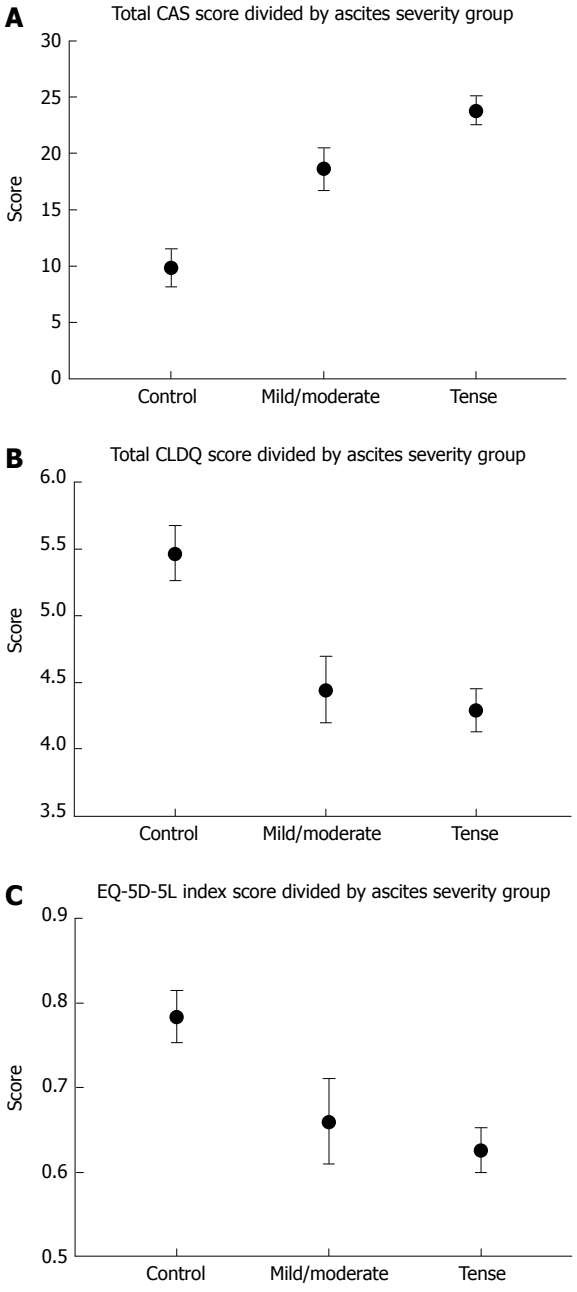


**Figure 2 Median test scores with SEM divided by control, mild/moderate and tense ascites respectively.** A: CAS scale. Control *vs* mild/moderate, $P < 0.001$; control *vs* tense, $P < 0.001$; B: CLDQ. Control *vs* mild/moderate $P = 0.002$; Control *vs* tense, $P < 0.001$; C: EQ-5D-5L. Control *vs* mild/moderate, $P = 0.038$; Control *vs* tense, $P = 0.002$. CAS: Cirrhosis-associated ascites symptom scale; CLDQ: Chronic liver disease questionnaire; EQ-5D-5L: EuroQoL 5-Dimensions 5-Level scale questionnaire.

multi-morbidity in cirrhosis; validated and recommended scores such as SF36 or CLDQ should be used, with the CAS scale as a supplement[9]. Consequently, CAS scale validation also requires further research on larger sample size and applied on standardized groups of subjects. An interesting study setup would be to test the CAS scale on patients with cirrhosis presented with ascites before and after treatment. Further, the CAS scale ought to be tested as a monitoring tool for efficacy in intervention trials, compared with CLDQ and SF-36 questionnaires.

In conclusion, the present construction and validation of the CAS scale seems promising for future research in the area of ascites symptom management and HRQL. Initially the scale requires testing in other study setups in order to clarify the quality and practicability of the questionnaire. In future perspectives an optimized and/or further validated version of the CAS scale can be used as a monitoring tool in interventional trials and to assess the effects of standardized treatment. Ultimately the CAS scale can contribute to gaining further knowledge on how to treat ascites effectively and consequently improve patients' quality of life.

## ARTICLE HIGHLIGHTS

### *Research background*

Cirrhosis is associated with a detrimental effect on health related quality of life (HRQL). Disease-specific questionnaires have been developed for the assessment of HRQL in patients with liver disease and cirrhosis. The questionnaires include aspects that are of particular relevance to patients with cirrhosis, such as concerns about complications and liver transplantation.

### *Research motivation*

No previous studies have specifically evaluated the association between the severity of ascites related symptoms and HRQL. However, it is likely that the severity of symptoms is important.

### *Research objectives*

We therefore developed and evaluated the predictive ability of a symptom assessment scale specifically made to assess symptoms related to cirhosis-associated ascites. We subsequently undertook a multicenter cohort study to evaluate the association between our scale and a disease specific scale (CLDQ) as well as a generic HRQL questionnaire (EQ-5D-5L).

### *Research methods*

Development of the cirrhosis-associated ascites symptom (CAS) scale included a literature review, and a qualitative study to evaluate face and content validity as well as discriminatory ability. We initially searched for previously validated scales in MEDLINE and EMBASE. The searches were combined with manual searches of reference lists in potentially relevant articles and conference proceedings. We then proceeded with a qualitative study, where ten independent experts identified and listed what they believed were the most important symptoms with an expected negative impact on HRQL. Symptoms should occur frequently or be severe enough to have substantial impact on daily function in order to be considered for our scale.

We then conducted open interviews followed by structured interviews of ten patients with cirrhosis and moderate ($n = 4$) or severe ascites ($n = 6$). The selected domains were initially presented and patients were asked to rate them according to the patient's perceived 'importance', on the extent to which the symptom had bothered them.

We revised the questionnaire instrument based on the answers.

After the development of the questionnaire, we prospectively enrolled a validation cohort consisting of patients with cirrhosis of any aetiology and severe, moderate or no ascites. All gave their informed consent to participate.

Subjects completed the following scales and questionnaires: CAS scale; Chronic Liver Disease Questionnaire; and EQ-5D-5L questionnaire.

We used the Crohnbach's alpha to assess equivalent reliability. *Discriminant validity* was evaluated using Dunnett's test to adjust for multiple comparisons. We originally planned to evaluate *convergent validity* with previously validated scales assessing symptoms or HRQOL associated with ascites. Since no previous scales were identified, we used the CLDQ and the EQ-5D-5L. We compared scale scores using Spearman correlation and included the CAS scale, CLDQ, CLDQ subscale parameters, and EQ-5D-5L. Correlations higher than 0.70 were considered strong and values below 0.40 were interpreted as poor.

### Research results

The final scale included 14 items. Chronbach's alpha was 0.88 for the total score, which we considered as acceptable. The validation cohort included 103 patients.

The proportion of patients with Child Pugh A was 24%, Child Pugh B: 58% and Child Pugh C: 19%. Fourty-four percent had severe ascites and 27 percent had moderate ascites. A control group of 30 percent had no ascites. The mean scores for each question in the CAS scale suggested that symptoms were worse for patients with severe ascites than for controls. The CAS scale score found that patients with severe ascites or moderate ascites had significantly worse scores compared with controls. Based on the CLDQ questionnaire, severe ascites had a detrimental impact on HRQOL compared with controls as did moderate ascites. The EQ-5D-5L also found a lower HRQOL in patients with severe or moderate ascites. We found a strong correlation between the CAS and the CLDQ total score as well as the CLDQ subscores; fatigue, activity and systemic symptoms.

### Research conclusions

This study has developed and tested a symptom assessment scale for the impact of ascites in cirrhosis. The CAS scale is easy to use and correlates well with more extensive QoL questionnaires.

### Research perspectives

This study has brought focus to the impact and importance of effective management of ascites in chronic liver disease.

The CAS scale should be tested in larger clinical and interventional trials, preferably in combination with CLDQ or other generic health related quality of life questionnaires.

The CAS scale should be tested in larger cohorts with various etiologies for chronic liver disease and ascites, also in malignant ascites. The CAS scale should also be tested in interventional trials in which the effect of a given intervention on the CAS scale is evaluated, to demonstrate whether the CAS scale is applicable as a monitoring tool in ascites.

## ACKNOWLEDGMENTS

The authors would like to thank the health care professionals at the Gastro Unit, Copenhagen University Hospital Hvidovre, who participated in the qualitative study, thereby contributing to the design of the CAS scale. We also wish to thank medical professor Per Bech and senior doctor Finn Zierau for valuable feedback on statistical issues.

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**P- Reviewer**: Jarcuska P, Jin B, Maruyama M, Niu ZS, Shimizu Y
**S- Editor**: Wang JL **L- Editor**: A **E- Editor**: Huang Y

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

*World J Gastroenterol* 2018 April 21; 24(15): 1658-1665

DOI: 10.3748/wjg.v24.i15.1658

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

META-ANALYSIS

# Platelet-to-lymphocyte ratio in the setting of liver transplantation for hepatocellular cancer: A systematic review and meta-analysis

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Author contributions: Lai Q contributed to conception and design of the study; Lai Q, Melandro F, Giovanardi F, Ferri F and Hassan R contributed to acquisition of data; Lai Q and Melandro F analysed and interpreted the data; Lai Q drafted the article; Ginanni Corradini S, Rossi M and Mennini G critically revised the manuscript; and all authors approved the final version.

Conflict-of-interest statement: The authors have no conflicts of interest to declare.

PRISMA 2009 Checklist statement: The authors have read the PRISMA 2009 Checklist, and the manuscript was prepared and revised according to the PRISMA 2009 Checklist.



Manuscript source: Invited manuscript

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Received: March 9, 2018
Peer-review started: March 10, 2018
First decision: March 29, 2018
Revised: April 2, 2018
Accepted: April 9, 2018
Article in press: April 9, 2018
Published online: April 21, 2018

## Abstract

### *AIM*

To perform a systematic review and meta-analysis on platelet-to-lymphocyte ratio (PLR) as a risk factor for post-transplant hepatocellular cancer (HCC) recurrence.

### *METHODS*

A systematic literature search was performed using PubMed. Participants of any age and sex, who underwent liver transplantation for HCC were considered following these criteria: (1) studies comparing pre-transplant low *vs* high PLR values; (2) studies reporting post-transplant recurrence rates; and (3) if more than one study was reported by the same institute, only the most recent was included. The primary outcome measure was set for HCC recurrence after transplantation.

### *RESULTS*

A total of 5 articles, published between 2014 and 2017, fulfilled the selection criteria. As for the quality of the reported studies, all the investigated articles presented

an overall high quality. A total of 899 cases were investigated: 718 cases (80.0%) were males. Three studies coming from European countries and one from Japan presented HCV as the main cause of cirrhosis. On the opposite, one Chinese study presented a greater incidence of HBV-related cirrhotic cases. In all the studies apart one, the PLR cut-off value of 150 was reported. At meta-analysis, high PLR value was associated with a significant increase in recurrence after transplantation (OR = 3.33; 95%CI: 1.78-6.25; $P < 0.001$). A moderate heterogeneity was observed among the identified studies according to the Higgins $I^2$ statistic value.

***CONCLUSION***

Pre-transplant high PLR values are connected with an increased risk of post-operative recurrence of hepatocellular cancer. More studies are needed for better clarify the biological mechanisms of this results.

**Key words:** Recurrence; Inflammation; Hepatocellular cancer; Liver transplantation; Platelet-to-lymphocyte ratio



**Core tip:** Poor data exist on the role of the inflammatory marker platelet-to-lymphocyte ratio (PLR) and hepatocellular cancer (HCC) recurrence after liver transplantation. This is the first systematic review and meta-analysis specifically investigating the role of PLR in the setting of liver transplant for HCC. Pre-transplant high PLR values confirmed their utility as predictors of recurrence, being connected with a 3.33-fold increased risk of post-transplant HCC recurrence.

Lai Q, Melandro F, Larghi Laureiro Z, Giovanardi F, Ginanni Corradini S, Ferri F, Hassan R, Rossi M, Mennini G. Platelet-to-lymphocyte ratio in the setting of liver transplantation for hepatocellular cancer: A systematic review and meta-analysis. *World J Gastroenterol* 2018; 24(15): 1658-1665 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i15/1658.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i15.1658

## INTRODUCTION

Liver transplantation (LT) represents the best therapy for the treatment of hepatocellular cancer (HCC)[1]. However, LT represents a scarce resource. As a consequence, a careful selection of HCC patients must be done preoperatively, with the intent to minimize the risk of post-LT recurrence[2] It is, in fact, clear that transplanting too advanced tumors is connected with a higher risk of poor post-LT outcomes[3]. Moreover, an error in the selection process corresponds to a "futile transplant", avoiding to transplant another patient in the waiting list[4].

After the introduction of the Milan Criteria (MC) in 1996, several other scores have been proposed in the last decades with the intent to refine the selection of HCC patients waiting for LT[5-8]. Apart from tumor morphology, also biology has been integrated into prognostic scores in the last years: Thus, the markers alpha-fetoprotein and des-gamma-carboxy-prothrombin, the radiological response after locoregional therapies or the tumor behaviour at PET scan have been largely investigated[9-13]. Recently, also systemic inflammation has been added as a possible value to add in the complex "mainframe" of HCC selection[14]. Among the different evaluated markers, the neutrophil-to-lymphocyte ratio (NLR) has been proposed as the most promising predictor of HCC recurrence[15,16]. NLR has been also integrated into several scores aimed at better select HCC patients waiting for LT[17,18]. However, another less intensely investigated ratio, namely the platelet-to-lymphocyte ratio (PLR), has also reported interesting results[19,20].

The main aim of the present study is to report a systematic review of the literature and a meta-analysis focused on investigating the role of PLR in the setting of liver transplantation as a useful predictor of HCC recurrence.

## MATERIALS AND METHODS

### *Search strategy*

A systematic search was done in relation to relevant studies focusing on the role of PLR in HCC patients undergoing LT. The search strategy was done in accordance with the Preferred Reporting Items for Systemic Reviews and Meta-Analysis (PRISMA) guidelines, as well as PRISMA for abstracts[21]. A search of the electronic databases MEDLINE-PubMed, Cochrane Library and EMBASE was conducted using the following research terms: (liver transplant*[tw]) AND (platelet-to-lymphocyte ratio[tw] OR PLR[tw]). Text word [tw] was preferred respect to MeSH words with the intent to identify In Process citations. Studies published before March 6, 2018, were taken into consideration.

### *Screening process*

The present qualitative systematic review included a priori search criteria of journal articles among adult (age $\geqslant$ 18 years) human patients. Studies were limited to the English language. We defined as enrollable all the studies based on HCC patients having received LT in which pre-operative PLR values were correlated with the risk of post-LT HCC recurrence. Investigated time to recurrence was set at 5 years after LT.

Exclusion criteria were: (1) papers lacking sufficient details; (2) review articles; (3) nonclinical studies; (4) expert opinions; (5) letters; (6) conference summaries; and (7) case reports.

### *Study selection*

Two reviewers (QL and FM) independently screened the identified studies and their extracted data. In case

**Table 1 Quality of studies evaluated by the modified Newcastle-Ottawa scale**

| Ref. | Selection | | | | Comparability | | Outcome | | Quality score |
|---|---|---|---|---|---|---|---|---|---|
| | Case definition | Representativeness | Selection of controls | Definition of controls | Comparable for therapy | Comparable for etiology | Assessment of outcomes | Integrity of follow-up | |
| Xia *et al*[24] | Yes | Yes | No | Yes | Yes | Yes | Yes | Yes | ★★★★ ★★★★ |
| Lai *et al*[25] | Yes | Yes | No | Yes | Yes | Yes | Yes | Yes | ★★★★ ★★★★ |
| Parisi *et al*[26] | Yes | Yes | No | Yes | Yes | Yes | Yes | Yes | ★★★★ ★★★★ |
| Nicolini *et al*[27] | Yes | Yes | No | Yes | Yes | Yes | Yes | Yes | ★★★★ ★★★★ |
| Harimoto *et al*[28] | Yes | Yes | No | Yes | Yes | Yes | Yes | Yes | ★★★★ ★★★★ |

of disagreement, the paper was discussed by all the authors.

### *Quality assessment*

Selected studies were reviewed based on the representativeness of the study population, comparability of cohorts, adequate assessment of outcomes, sufficient length of follow-up, adequacy of follow-up, and source of study funding. The quality of the papers was assessed using the Newcastle-Ottawa Quality Assessment Scale (NOS): studies with scores > 6 were defined as high-quality studies[22].

NOS details of each selected study were reported in Table 1. The characteristics coming from each study were collected in Table 2. The following features were collected: first author's name, reference number, number of patients, patient age, patient gender, waiting time duration in months, model for end-stage liver disease, underlying liver pathology, the diameter of the major lesion and the number of tumors at the moment of LT, the MC-OUT status at the moment of LT, AFP value ⩾ 200 ng/mL, any type of locoregional treatment (LRT), the PLR cut-off used in the article, the area under the receiver operator curve (ROC) for the diagnosis of recurrence, the number of post-LT recurrences and the 5-year tumor-free survival (TFS).

### *Statistical analysis*

Different PLR cut-offs were observed among the identified studies. TFS end-point in the different studies corresponded to 5 years after LT. Summary measures were extracted from each study and used to generate a pooled odds ratio (OR). Higgins $I^2$ statistic was used to assess heterogeneity. Higgins $I^2$ statistic values of 0%-25%, 25%-50%, and > 50% were considered as indicative of homogeneity, moderate heterogeneity, and high heterogeneity, respectively. Only the random-effects model was used, starting from the assumption that a common OR was unreliable in the analyzed studies due to the broad eligibility criteria and the different used PLR cut-off values. OR was considered statistically significant when the *P*-value was < 0.05. OR and 95% confidence intervals (CI) > 1 revealed that the patients with high PLR values had poor prognoses (higher risk of recurrence), whereas a result < 1 had the opposite meaning. The analysis was performed using OpenMEE software (http://www.cebm.brown.edu/openmee/index.html).

## RESULTS

The selection process of the articles is explained in Figure 1.

As for the selection process according to the PRISMA guidelines, the various examined databases provided a total of 39 articles to screen. After removing the duplicates and reading the title and the abstract, 28 articles were removed. Of the remaining 11 papers, 6 were not considered eligible after full-text evaluation. Two studies coming from Hangzhou China were performed on the same population, so only one of these studies was selected for the last analysis[23,24].

Eventually, 5 articles were identified, with a total of 899 investigated cases (Table 2)[24-28].

As for the quality of the reported studies, all the investigated articles were retrospective cohort studies all presenting the excellent NOS value of eight, thus reporting the overall high quality of the studies focused on this topic (Table 1).

In the selected series, median/mean age ranged 49-58 years. As for patient gender, 718 cases (80.0%) were males. Three studies coming from European countries and one from Japan presented HCV as the main reason for underlying cirrhotic liver disease (298/556 cases; 53.6%). On the opposite, one Chinese study presented a greater incidence of HBV-related cirrhotic cases (320/343 cases; 93.3%).

Last radiology before LT was available in only three studies, showing a median diameter of the major lesion ranging 1.3-2.7 cm and a median single lesion (range 0-2). MC-OUT status was observed in 309 (34.4) patients: interestingly enough, a great discrepancy was observed among the reported studies, with one series coming from Europe showing no cases exceeding the MC, and the study coming from China presenting 58.0% of MC-OUT individuals. Different AFP cut-offs

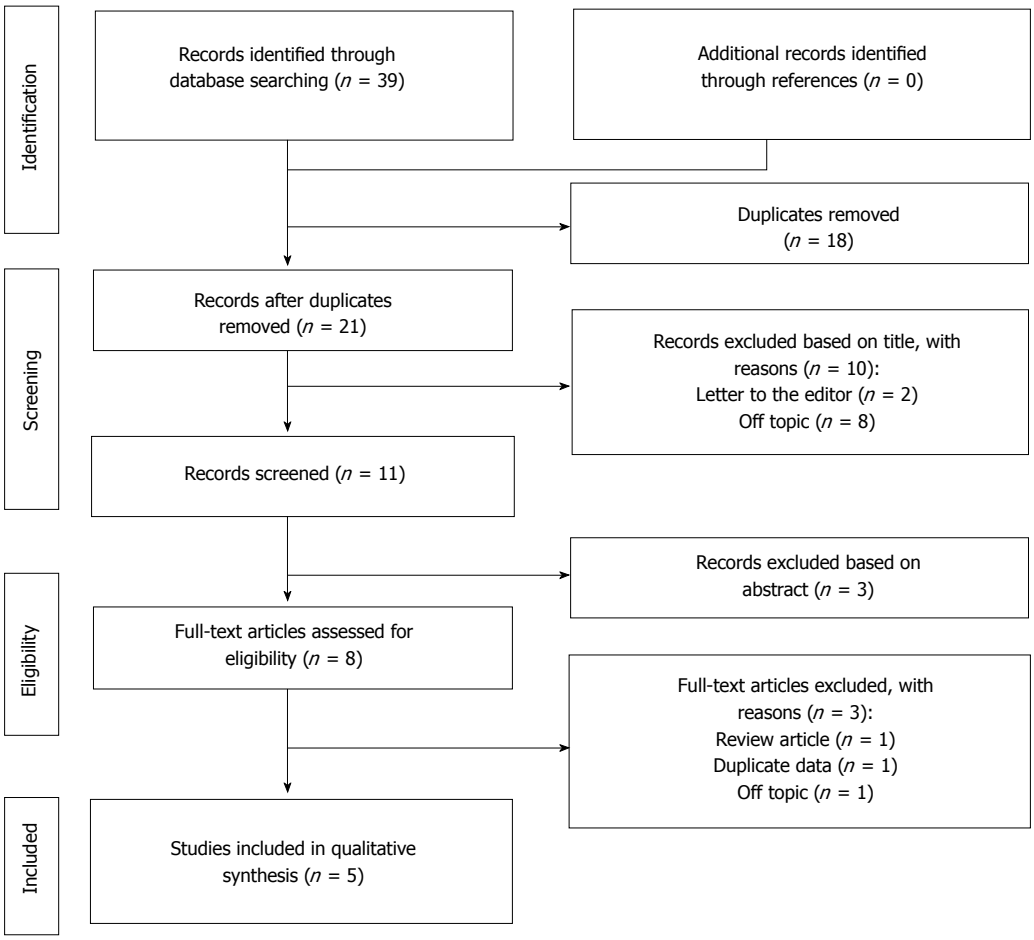


**Figure 1 PRISMA flowchart of the literature search and study selection.**

were reported in the studies (200/300/400 ng/mL). Also in this case, great discrepancies were observed among the study coming from Hangzhou and the other ones in terms of cases exceeding the reported threshold values (48.1% *vs* 5.5%-15.8%). When reported, LRT were performed in 47.3%-100.0% of cases.

Specifically investigating the PLR values, the cut-off of 150 was reported in all the series apart from the study from Fukuoka Japan, in which the median value of 70.4 was investigated. When the diagnostic power of PLR in terms of HCC recurrence was investigated, an area under the ROC curve of 0.63-0.70 was observed, showing an acceptable-to-good ability of this variable to diagnose post-transplant recurrence. A total of 180 (20.0%) cases exceeded the proposed threshold values, with a percentage ranging 7.1%-51.1% in the various series.

Five-year tumor-free survivals were reported in four studies. In all of them, patients presenting high PLR values had worse results, with a value ranging 81%-25% respect to the ones reported in patients with low PLR values (95%-52%).

The binary random-effects meta-analysis showed a strong relationship between poor TFS and elevated PLR values (OR = 3.33, 95%CI: 1.78-6.25; $P < 0.001$). Higgins $I^2$ statistic presented a value = 26.8% ($P$ = 0.24), showing a moderate heterogeneity among the examined studies (Figure 2).

## DISCUSSION

Until now, few data have been reported on the predictive role of PLR as a risk factor for HCC recurrence after liver transplant. Indeed, only five studies have been identified in the present systematic review, the first of whom published in 2014[24-28]. However, although the number of reported cases is relatively limited ($n$ = 899), the biological effect of PLR looks to be clear, with a strong correlation between high PLR values and a greater risk for recurrence. According to the results of the meta-analysis, subjects having pre-LT high PLR values present a 3.33-fold increased risk of experiencing HCC recurrence after LT.

Interestingly enough, the only study in which the PLR failed to be a prognostic tool for recurrence was the sole in which only patients meeting the MC were transplanted[26]. Such an evidence should represent a possible explanation for the observed results. It is, in fact, possible that a direct correlation may exist between higher PLR values and a progressively increasing tumor aggressiveness (*i.e.*, higher AFP and greater tumor burden). As a possible confirmation of

**Table 2 Demographic and clinical aspects of the selected studies**

| Ref. | *n* | Age | Male gender (%) | waiting time (mo) | MELD | Underlying disease | Major lesion diam (cm) | Number lesions | MC-OUT (%) | AFP ⩾ 200 ng/mL (%) | LRT (%) | Cut-off | AUROC | > cut-off (%) | Recurr (%) | 5-yr TFS |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Xia *et al*[24] | 343 | 49 ± 10 | 308 (90) | NA | 13 ± 6 | HBV = 320<br>Other = 23 | > 5:110 | > 3:91 | 199 (58) | 165 (48) | 222 (65) | 150 | 0.63 | 33 (10) | NA | < 150: 52<br>⩾ 150:25 |
| Lai *et al*[25] | 146 | 58 (54-63) | 116 (80) | 8 (3-10) | 11 (8-11) | HCV = 63<br>HBV = 26<br>Other = 57 | 2.5 (1.7-3.5) | 1 (1-2) | 32 (22) | 8 (6) | 136 (93) | 150 | 0.66 | 28 (19) | 14 (10) | < 150:92<br>⩾ 150:81 |
| Parisi *et al*[26] | 150 | 54 ± 7 | 125 (83) | 2 (0-12) | NA | HCV = 60<br>HBV = 34<br>Other = 56 | 2.7 | 1 | 0 (-) | 13 (9) | 71 (47) | 150 | NA | 17 (11) | 19 (13) | NA |
| Nicolini *et al*[27] | 70 | 57 (51-62) | 62 (89) | NA | 11 (7-15) | HCV = 41<br>HBV = 15<br>Other = 14 | 1.3 (0.0-2.1) | 1 (0-2) | 12 (17) | 6 (9)[1] | 70 (100) | 150 | NA | 5 (7) | 8 (11) | < 150:89<br>⩾ 150:50 |
| Harimoto *et al*[28] | 190 | ⩾ 59:97 | 107 (56) | NA | ⩾ 15:60 | HCV = 134<br>Other = 56 | > 5:8 | > 3:41 | 66 (35) | 30 (16)[2] | NA | 70.4 | 0.70 | 97 (51) | 28 (15) | < 70.4:95<br>⩾ 70.4:76 |

[1]AFP ⩾ 400 ng/mL; [2]AFP ⩾ 300 ng/mL. MELD: Model for end-stage liver disease; MC: Milan Criteria; AFP: Alpha-fetoprotein; LRT: Locoregional treatment; AUROC: Area under the receiver operator curve; TFS: Tumor-free survival; HCV: Hepatitis C virus; HBV: Hepatitis B virus; NA: Not available.

these data, all the series reported in the present study showed a gradient among PLR values, patients exceeding the MC and worse survival results.

This evidence has been observed also in an ITA.L.I.C.A. study in which a direct correlation between tumor dimension and the absolute number of platelets was reported[29]. Another study from Taiwan performed on more than 3000 HCC patients showed that platelets count efficaciously predicted extrahepatic metastases, even better than AFP did[30].

The link between HCC and platelets has been recently documented also in a Korean study only investigating absolute platelets count and HCC recurrence. A platelets value > 75 × $10^9$/L was connected with higher 5-year recurrence rates (28.2% *vs* 13.2% in patients with lower count; $P = 0.002$). Similarly, at multivariable analysis, a significantly greater recurrence risk was confirmed in the high platelets group (HR = 1.90; 95%CI: 1.02-3.54; $P = 0.04$). Of interest, platelets count remained significant as a risk factor for recurrence even when it was introduced in a multivariable model comprehending aspects of tumor biology and morphology. Thus, we can postulate that platelets present an independent role in favouring tumor progression[31].

As a confirmation of this result, it has been well established that platelets are effector cells directly interacting with tumor cells in the metastatic cascade[32,33]. During total hepatectomy for LT, some tumor cells may be observed in the bloodstream due to HCC manipulation, only needing a few hours to days to complete the metastatic cascade[33]. Platelets favour some of the mechanisms required for metastatic dissemination: For example, they favour tumor cell surviving in the bloodstream, extravasation, initial seeding and tumor re-growth[34].

All these considerations present great repercussions from a clinical point of view: In fact, platelets might provide a potential therapeutic target for anti-hepatoma treatments. Of interest, sorafenib already represents an anti-HCC drug directly acting against cellular pathways mediated by platelet-derived growth factors (*i.e.*, vascular endothelial growth factor and platelet-derived growth factor)[35]. Other therapies including cyclooxygenase inhibitors, protease-activated receptor inhibitors, and glycoprotein IIb/IIIa inhibitors may further play an underestimated role in this phenomenon[36].

However, although the reported data suggest an effective biological correlation between platelets and tumor aggressive behaviour, we should underline that further clinical studies trying to univocally demonstrate the biological role of platelets in the HCC oncogenesis are needed. Indeed, the present meta-analysis was in fact affected by several potential shortcomings. First, moderate heterogeneity was observed among the studies investigated, as clearly shown by the reported Higgins $I^2$ statistic value (26.8%). Such a phenomenon was surely caused by the broad eligibility criteria for HCC and the different PLR cut-off values used in the different centers. It is, in

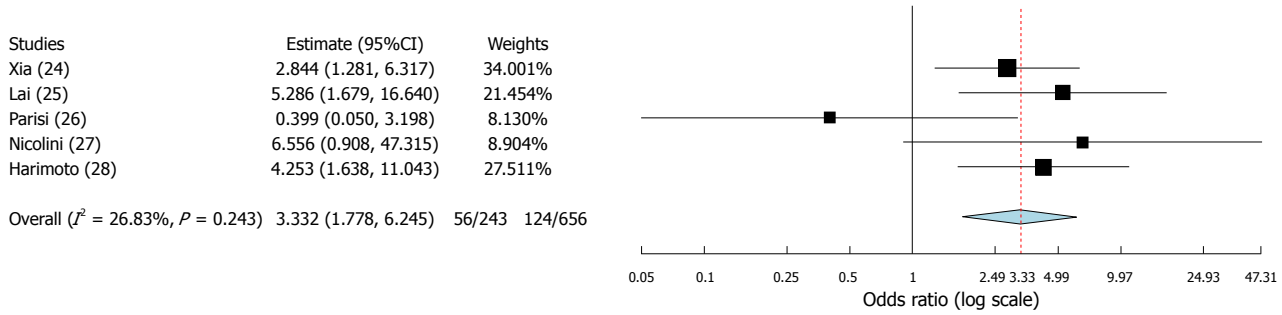


**Figure 2 Forest plot of odds ratios and 95% confidence intervals for the association between platelet-to-lymphocyte ratio and recurrence in hepatocellular cancer patients undergoing liver transplantation.** Weights are from binary random-effect analysis.

fact, clear that a meta-regression weighted for the geographical area, HCV *vs* HBV as the main cause of liver failure, living-donor *vs* deceased-donor LT, and markers of tumor aggressiveness should represent a more accurate way for better clarify the role of PLR in this setting. Unfortunately, the limited number of cases reported did not consent us to perform more sophisticated analyses. Secondly, no information was reported in the different series on the presence and grade of portal hypertension, a very well known cause of thrombocytopenia in cirrhosis[37].

In conclusion, platelet-to-lymphocyte ratio is an easy and cheap value to use for selecting patients with hepatocellular cancer waiting for liver transplantation. A direct correlation between PLR values and tumor aggressiveness has been observed in several studies. High pre-transplant PLR values cause a 3.3-fold increased risk for post-transplant recurrence. More studies aimed at better understanding biological and clinical mechanisms of the link between PLR and HCC are needed.

## ARTICLE HIGHLIGHTS

### Research background

Liver transplantation is the best curative therapy in case of hepatocellular cancer (HCC). However, it represents a scarce resource due to the reduced number of donors. Thus, a careful selection of HCC patients must be done preoperatively, with the intent to minimize the risk of futile transplants (*i.e.*, post-operative cancer recurrence). As a consequence, new and easy-to-use predictors of recurrence are needed.

### Research motivation

Recently, several biological aspects of HCC have been investigated, with the intent to identify scores aimed at improving the prediction of poor post-transplant outcomes. Among them, the inflammatory marker platelet-to-lymphocyte ratio (PLR) has been only marginally investigated, although it should represent a potentially excellent and cheap marker to use.

### Research objectives

The main objective of the present study is to evaluate the role of PLR as a possible selection tool for the risk of HCC recurrence in the setting of liver transplantation.

### Research methods

A systematic review and a meta-analysis have been performed with the intent to evaluate the role of PLR. The PRISMA Guidelines have been used for performing the systematic research of studies focused on PLR, HCC and LT.

### Research results

Five articles coming from Europe and Asia have been identified, with a total of 899 subjects investigated. At meta-analysis, high PLR value was associated with a significant increase in recurrence after transplantation (OR = 3.33; 95%CI: 1.78-6.25; $P$ < 0.001).

### Research conclusions

A direct correlation between PLR values and tumor aggressiveness has been observed. High pre-transplant PLR values cause a 3.3-fold increased risk for post-transplant recurrence. Platelet-to-lymphocyte ratio is an easy and cheap value to use for selecting patients with hepatocellular cancer waiting for liver transplantation. PLR should be taken into account in the creation of new selection scores for HCC.

### Research perspectives

More studies aimed at better understanding biological and clinical mechanisms of the link between PLR and HCC are necessary.

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**P- Reviewer**: Boin IF, Gencdal G, Ramia JM, Zheng SS
**S- Editor**: Gong ZM **L- Editor**: A **E- Editor**: Huang Y

World Journal of Gastroenterology

Submit a Manuscript: http://www.f6publishing.com

*World J Gastroenterol* 2018 April 21; 24(15): 1666-1678

DOI: 10.3748/wjg.v24.i15.1666

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

META-ANALYSIS

# Impact of enhanced recovery after surgery programs on pancreatic surgery: A meta-analysis

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Author contributions: Zhu WT, Wei Q, and Chen QP designed the research; Ji HB, Wei Q, Wang XX, and Wang HB performed the research; Ji HB, Zhu WT, and Wei Q analyzed the data; Ji HB, Wang XX, and Chen QP wrote the paper.

Conflict-of-interest statement: The authors deny any conflict of interest.

PRISMA 2009 Checklist: The authors have read and revised according to the PRISMA 2009 Checklist.



Manuscript source: Unsolicited manuscript.

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Received: February 5, 2018
Peer-review started: February 6, 2018
First decision: February 24, 2018
Revised: March 8, 2018
Accepted: March 18, 2018
Article in press: March 18, 2018
Published online: April 21, 2018

## Abstract

***AIM***

To evaluate the impact of enhanced recovery after surgery (ERAS) programs on postoperative complications of pancreatic surgery.

***METHODS***

Computer searches were performed in databases (including PubMed, Cochrane Library and Embase) for randomized controlled trials or case-control studies describing ERAS programs in patients undergoing pancreatic surgery published between January 1995 and August 2017. Two researchers independently evaluated the quality of the studies' extracted data that met the inclusion criteria and performed a meta-analysis using RevMan5.3.5 software. Forest plots, demonstrating the outcomes of the ERAS group *vs* the control group after pancreatic surgery, and funnel plots were used to evaluate potential publication bias.

***RESULTS***

Twenty case-control studies including 3694 patients, published between January 1995 and August 2017, were selected for the meta-analysis. This study included the ERAS group ($n$ = 1886) and the control group ($n$ = 1808), which adopted the traditional perioperative management. Compared to the control group, the ERAS group had lower delayed gastric emptying rates [odds ratio (OR) = 0.58, 95% confidence interval

(CI): 0.48-0.72, $P < 0.00001$], lower postoperative complication rates (OR = 0.57, 95%CI: 0.45-0.72, $P < 0.00001$), particularly for the mild postoperative complications (Clavien-Dindo Ⅰ-Ⅱ) (OR = 0.71, 95%CI: 0.58-0.88, $P = 0.002$), lower abdominal infection rates (OR = 0.70, 95%CI: 0.54-0.90, $P = 0.006$), and shorter postoperative length of hospital stay (PLOS) (WMD = -4.45, 95%CI: -5.99 to -2.91, $P < 0.00001$). However, there were no significant differences in complications, such as, postoperative pancreatic fistulas, moderate to severe complications (Clavien-Dindo Ⅲ-Ⅴ), mortality, readmission and unintended reoperation, in both groups.

***CONCLUSION***

The perioperative implementation of ERAS programs in pancreatic surgery is safe and effective, can decrease postoperative complication rates, and can promote recovery for patients.

**Key words:** Pancreatic surgery; Enhanced recovery after surgery; Postoperative complication; Meta-analysis



**Core tip:** Enhanced recovery after surgery (ERAS) programs have been launched in a variety of surgical fields, including colorectal, orthopedics, urology, esophageal and gynecology, demonstrating favorable outcomes. Pancreatic surgery is considered a high-risk abdominal surgery, due to increased surgical trauma and high incidence of postoperative complications. In this meta-analysis we aimed to evaluate the impact of ERAS on complications of pancreatic surgery. The present study demonstrates that ERAS could reduce complication rates, especially of mild complications, delayed gastric emptying, abdominal infection and postoperative length of hospital stay, while not affecting the rates of postoperative pancreatic fistulas, reoperation, readmission and mortality during the perioperative period.

Ji HB, Zhu WT, Wei Q, Wang XX, Wang HB, Chen QP. Impact of enhanced recovery after surgery programs on pancreatic surgery: A meta-analysis. *World J Gastroenterol* 2018; 24(15): 1666-1678 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i15/1666.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i15.1666

## INTRODUCTION

Enhanced recovery after surgery (ERAS; also called 'fast-track surgery') was first introduced by Kehlet H, a Danish surgeon, in 1997[1]. ERAS is a multidisciplinary and evidence-based framework developed to decrease perioperative surgical stress, accelerate postoperative recovery and significantly reduce the postoperative length of hospital stay (PLOS). ERAS programs were initially implemented in colorectal surgery and have been shown to be effective for reducing PLOS and complications[2]. Subsequently, ERAS programs have been published in numerous areas of surgery, such as orthopedics, urology, esophageal, gynecology, breast and hepatobiliary[3-8].

An array of studies has shown that the perioperative implementation of ERAS programs can reduce PLOS without increasing complications or mortality. However, pancreatic surgery is still considered a high-risk abdominal surgery, due to the anatomical location of the pancreas and high rate of complications (30%-60%). Postoperative complications, such as postoperative pancreatic fistula (POPF), delayed gastric emptying (DGE), abdominal infection, and so on, are the main reasons for delayed recovery and the frequent need for additional interventions, without which the complications are potentially life threatening. For these reasons, the implementation of ERAS programs has lagged for pancreatic surgeries.

There had been an increasing number of ERAS programs implemented in pancreatic surgery when the ERAS group published evidence-based consensus recommendations for pancreatic surgery in 2012[9]. The benefit of implementing ERAS programs on postoperative complications in pancreatic surgery has not reached consensus. For this reason, we performed a meta-analysis of the available studies on ERAS programs compared with traditional perioperative management in patients undergoing pancreatic surgery.

## MATERIALS AND METHODS

### *Search strategy*

A search was performed by two researchers (Ji HB and Wang XX) in August 2017 of the PubMed, Cochrane Library and Embase database, spanning the period from January 1995 to August 2017. The search language was restricted to English, using the search terms "enhanced recovery after surgery", "fast track surgery", "ERAS", "clinical pathways", "pancreatectomy", "pancreatoduodenectomy" and "duodenopancreatectomy", and using the Boolean operators "AND" and "OR". Synonyms of all these terms were used in this search. The PubMed search strategy for the meta-analysis is shown in Table 1.

### *Inclusion/exclusion criteria*

Studies meeting all of the following selection criteria were eligible for inclusion: (1) studies concerning patients undergoing pancreatic surgery; (2) the ERAS group implemented ERAS programs management, and the control group adopted traditional perioperative management; (3) measures in perioperative management were described in both groups; and (4) studies reported at least the following outcome measures, POPF, DGE, abdominal infection, mortality and PLOS, and explained their diagnostic criteria for postoperative complications.

**Table 1 The search strategy for the PubMed database[1]**

| Search number | Description | Number of publications |
|---|---|---|
| 1 | Enhanced recovery after surgery [Title/Abstract] OR ERAS [Title/Abstract] OR fast track surgery [Title/Abstract] | 3333 |
| 2 | Clinical pathways [MeSH Terms] | 5848 |
| 3 | 1 OR 2 | 9130 |
| 4 | Pancreatectomy [MeSH Terms] OR Pancreatectomy* [Title/Abstract] OR Pancreatoduodenectomy [MeSH Terms] OR Pancreatoduodenectom* [Title/Abstract] OR duodenopancreatectomy [MeSH Terms] OR duodenopancreatectom* [Title/Abstract] | 21497 |
| 5 | 3 AND 4 NOT (animals[mh] NOT humans[mh]) | 69 |
| 6 | 5 limited to English | 68 |

[1]Date of search: August 1, 2017.

Exclusion criteria were (1) sample size of less than 10; (2) comments, guidelines, reviews, case reports, abstracts, letters and non-comparative studies; (3) repeated publication of the same study population; and (4) incomplete clinical data.

### Outcomes of interest

The outcomes of interest were POPF, DGE, PLOS, abdominal infection, mortality, readmission, unintended reoperation and occurrence of any complication within a postoperative period of 30 d. POPF was defined using the International Study Group of Pancreatic Fistula (ISGPF) guidelines describing a drain output of any measurable volume of fluid on or after postoperative day (POD) 3, with an amylase content greater than three times the serum amylase activity or as defined by the study's authors[10]. DGE was defined according to the International Study Group of Pancreatic Surgery's (ISGPS) recommendation that patients needing maintenance of a nasogastric tube (NGT) for > 3 d, needing to reinsert the NGT for persistent vomiting after POD 3, or unable to tolerate a solid diet by POD 7, should be considered DGE. In addition, there are another two widely used definitions for DGE after pancreatic resection (1) Yeo defined DGE as an NGT left in place for ≥ 10 d plus one of the following, or for < 10 d plus two of the following (a) repeated emesis after removal of the NGT, (b) need for prokinetic agents after POD 10, (c) need for reinsertion of the NGT, or (d) failure to progress with the diet. (2) Van Berge Henegouwen *et al*[11] defined DGE as gastric stasis requiring NGT for ≥ 10 d or the inability to tolerate a regular diet after POD 14. PLOS was defined as the span from the day of surgery to the day of actual discharge from the hospital. Abdominal infection was defined by the study's authors. Mortality was defined as the range from the day of hospitalization to the first 30 d after actual discharge. Readmission was defined as the patient needing medical attention again within 30 d after discharge. Overall postoperative complications included any complication from the time of surgery to discharge, or within 30 d, with severity grading and classification relying on the Clavien-Dindo system[12]. Unintended reoperation was defined as patients with complications or other reasons that required reoperation within 30 d after discharge.

### Data extraction

Data were extracted from each study by two authors (Ji HB and Wei Q) independently. The main parameters included common information (time of study publication, country, study type, and authors), characteristics of the study population (sex and age), elements of ERAS programs, and postoperative outcomes (overall complications, POPF, DGE, abdominal infection, PLOS, mortality, readmission, and unintended reoperation). All continuous outcome variables were described using the means and standard deviations for this meta-analysis. We needed to estimate means and standard deviations via the methodologies reported by Hozo *et al*[13] if the original data were expressed as medians or ranges.

### Quality assessment

The quality assessment of each study was done by two authors (Zhu WT and Ji HB) independently via the Methodological Index for Non-Randomized Studies (MINORS) checklist. It was then summarized by a French surgeon, and if there was a disagreement, the third researcher was involved in the negotiation or adjudication, until a consensus was achieved. The MINORS checklist includes eight methodological items for non-comparative studies and an additional four items for comparative studies. The items are scored 0 (not reported), 1 (reported but inadequate), or 2 (reported and adequate). The overall ideal scores were 24 for comparative studies.

### Statistical analysis

The meta-analysis was performed using RevMan5.3.5 software (Ji HB and Wang HB). Continuous and categorical variables were calculated as weighted mean differences (WMDs) or odds ratios (ORs) with their corresponding 95% confidence interval (CI), respectively. Heterogeneity was assessed using a chi-square test, where $P > 0.05$ was considered non-significant. $I^2$ values were used for the evaluation of statistical heterogeneity, and the $I^2$ value of 50% or more indicated the presence

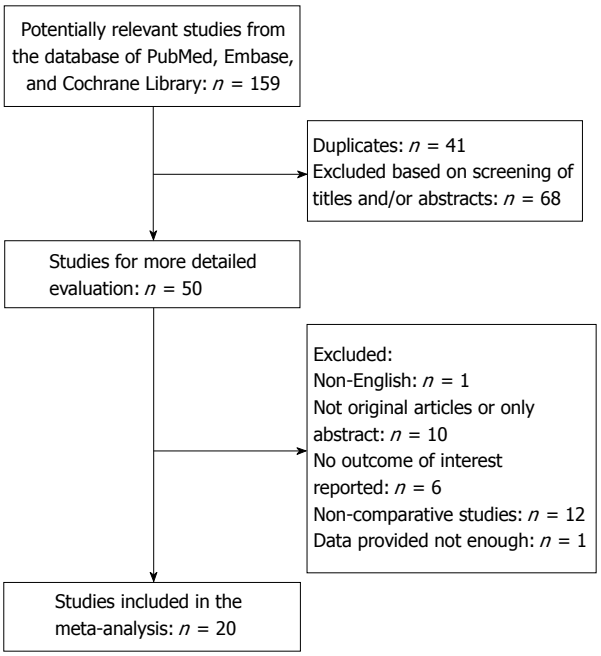


**Figure 1 The diagram of selected studies for this meta-analysis.**

of heterogeneity. The fixed-effects model was used for studies of homogeneity ($I^2$ < 50%), and the random-effects model was applied when studies indicated heterogeneity ($I^2 \geqslant$ 50%). In addition, funnel plots were used to evaluate potential publication bias based on the incidence of POPF and mortality.

### *Eligible studies*

The search strategy initially identified 159 relevant studies. No randomized control trials were identified. Figure 1 shows the process of selecting the studies for meta-analysis. After removing duplicates, the titles and abstracts of 118 studies were reviewed. Of these, 68 studies were not related to ERAS in pancreatic surgery, 12 studies did not have a control group, 6 studies did not have the outcomes of interest reported, 10 studies only had an abstract or we were unable to get the full text, 1 study did not have enough data, and 1 study was published in a language other than English. A total of 20 studies met the inclusion criteria for the meta-analysis.

### *Study characteristics and quality assessment*

The characteristics and quality assessments of the included studies are shown in Table 2[14-33]. All studies clearly described an ERAS program. The major components are summarized in Table 2. All of the studies used a retrospective case-control model, and of those, there were 16 studies that had sample sizes greater than 100. A total of 3694 patients were included, of which there were 1886 patients and 1808 patients included in the ERAS group and control group, respectively. In addition, there were 17 studies with MINORS scores > 12.

## RESULTS

### *Pancreatic fistula*

Eighteen studies reported the rates of POPF. The overall results (OR = 0.87, 95%CI: 0.74-1.03, *P* = 0.10; Figure 2), or only those using the ISGPF definition (OR = 0.90, 95%CI: 0.76-1.07, *P* = 0.24), showed that there were no significant differences present in either group. Furthermore, there was no significant difference in A (OR = 1.05, 95%CI: 0.81-1.36, *P* = 0.71), B (OR = 1.13, 95%CI: 0.85-1.51, *P* = 0.40), and C (OR = 0.90, 95%CI: 0.60-1.33, *P* = 0.59) grade of POPF between the ERAS group and control group.

### *DGE*

Eighteen studies reported the rates of DGE. Compared to the control group, the ERAS group had a lower incidence of DGE (OR = 0.58, 95%CI: 0.48-0.72, *P* < 0.00001; Figure 3). The difference persisted when including only studies that adopted the ISGPS definition (OR = 0.50, 95%CI: 0.39-0.65, *P* < 0.00001).

### *Postoperative complications*

The rate of overall postoperative complications was lower in the ERAS group (OR = 0.57, 95%CI: 0.45-0.72, *P* < 0.00001; Figure 4). Additionally, the incidence of mild postoperative complications (Clavien-Dindo I-II), which relies on the Clavien-Dindo definition of severity and classification, was lower in the ERAS group (OR = 0.71, 95%CI: 0.58-0.88, *P* = 0.002; Figure 5). There were no statistical differences in the moderate to severe complication rates (Clavien-DindoⅢ-Ⅴ) between the ERAS group and control group (OR = 0.90, 95%CI: 0.73-1.11, *P* = 0.32).

### *Abdominal infection*

A total of 12 studies reported the rates of abdominal infection. The incidence of abdominal infection was lower (OR = 0.70, 95%CI: 0.54-0.90, *P* = 0.006; Figure 6) in the ERAS group.

### *PLOS*

A total of 13 studies reported the PLOS, and they showed that the ERAS group had shorter PLOS (WMD = -4.45, 95%CI: -5.99 to -2.91, *P* < 0.00001; Figure 7) than the control group.

In addition, there were no significant differences in rates of mortality (OR = 0.85, 95%CI: 0.54-1.36, *P* = 0.51; Figure 8), readmission (OR = 1.04, 95%CI: 0.83-1.30, *P* = 0.75; Figure 9), and unintended reoperation (OR = 0.87, 95%CI: 0.63-1.20, *P* = 0.40; Figure 10).

### *Subgroup analysis*

The subgroup analysis, which included only larger

**Table 2 Study Characteristics and Quality Assessment.**

| Study | Year | Country | Study design | Sample size | | ERAS programs[1] | MINORS Score |
|---|---|---|---|---|---|---|---|
| | | | | ERAS group | Control group | | |
| Kennedy *et al*[14] | 2007 | United States | Case-control | 91 | 44 | e, f, g, h | 16/24 |
| Vanounou *et al*[15] | 2007 | United States | Case-control | 145 | 64 | c, d, g, h | 15/24 |
| Balzano *et al*[16] | 2008 | Italy | Case-control | 252 | 252 | d, e, f, g, h | 13/24 |
| Kennedy *et al*[17] | 2009 | United States | Case-control | 71 | 40 | d, e, f, g, h | 11/24 |
| Abu Hilal *et al*[18] | 2013 | Britain | Case-control | 20 | 24 | b, e, f, g, h | 15/24 |
| Braga *et al*[19] | 2014 | Italy | Case-control | 115 | 115 | a, b, c, d, e, f, g, h | 17/24 |
| Pillai *et al*[20] | 2014 | India | Case-control | 20 | 20 | c, d, e, f, g, h | 17/24 |
| Coolsen *et al*[21] | 2014 | Holland | Case-control | 86 | 97 | b, c, d, e, f, g, h | 12/24 |
| Nussbaum *et al*[22] | 2014 | United States | Case-control | 100 | 142 | c, e, f, g, h | 11/24 |
| Yui *et al*[23] | 2014 | Japan | Case-control | 57 | 52 | e, g, h | 13/24 |
| Nussbaum *et al*[24] | 2014 | United States | Case-control | 50 | 100 | c, e, f, g, h | 16/24 |
| Kobayashi *et al*[25] | 2014 | Japan | Case-control | 100 | 90 | a, e, g, h | 13/24 |
| Shao *et al*[26] | 2015 | China | Case-control | 325 | 310 | d, e, f, g, h | 15/24 |
| Joliat *et al*[27] | 2015 | Switzerland | Case-control | 74 | 87 | a, b, c, d, e, f, g, h | 15/24 |
| Partelli *et al*[28] | 2015 | Italy | Case-control | 22 | 66 | a, c, d, e, f, g, h | 13/24 |
| Williamsson *et al*[29] | 2015 | Sweden | Case-control | 50 | 50 | c, d, e, f, g, h | 17/24 |
| Morales Soriano *et al*[30] | 2015 | Spain | Case-control | 41 | 44 | a, b, c, d, e, f, g, h | 17/24 |
| Bai *et al*[31] | 2016 | China | Case-control | 124 | 63 | a, d, e, f, g, h | 15/24 |
| Zouros *et al*[32] | 2016 | Greece | Case-control | 75 | 50 | a, b, c, d, e, f, g, h | 16/24 |
| Dai *et al*[33] | 2017 | China | Case-control | 68 | 98 | a, b, c, e, f, g, h | 15/24 |

[1]ERAS programs: a: No bowel preparation in the preoperative period; b: Clear fluids until 2-3 h before surgery; c: Restrictive policy of intravenous fluids in the intra-operative period; d: Multimodal analgesia of the postoperative period; e: Clear fluids or food intakes in the early period; f: Enhanced mobilization in the early period; g: Removal of the drainage tube; h: Others. ERAS: enhanced recovery after surgery; MINORS score: Methodological Index for Non-Randomized Studies checklist.

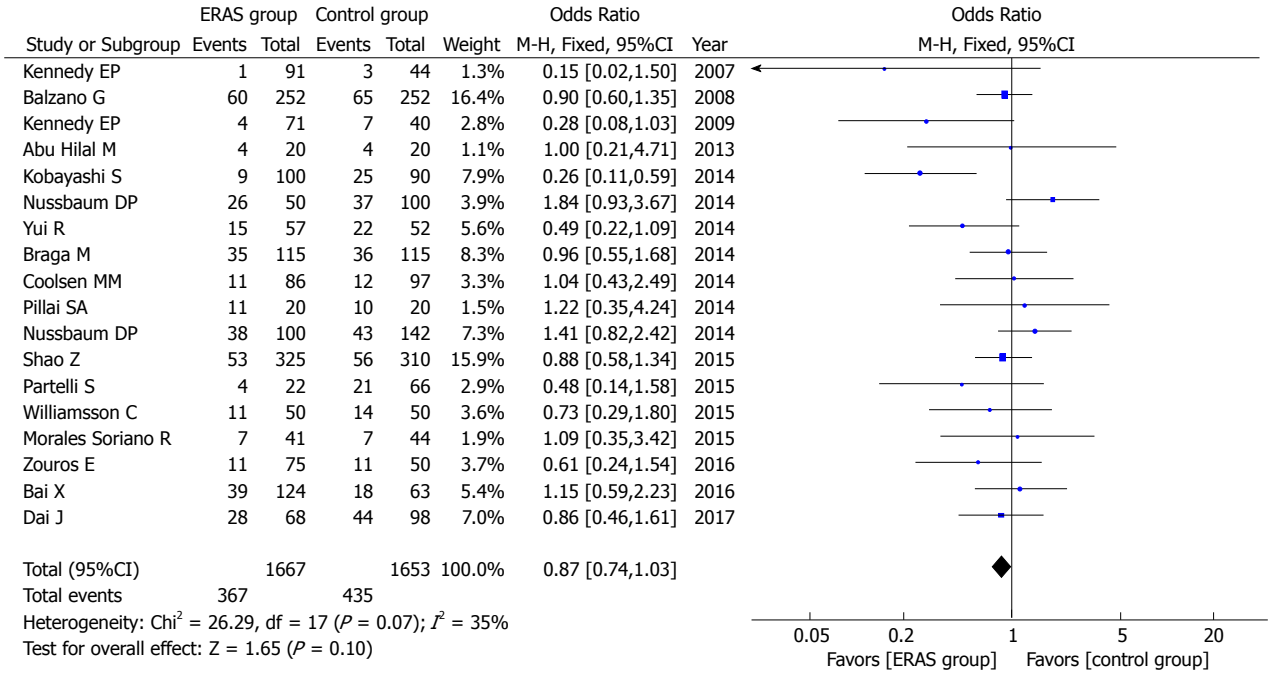


**Figure 2 Forest plots demonstrating the outcomes of postoperative pancreatic fistula.**

size studies ($n \geqslant 100$) generated similar results in postoperative outcomes (Table 3). Furthermore, the analysis of only high-quality studies (MINORS score > 12) also yielded parallel results in postoperative outcomes (Table 3). However, the heterogeneity for overall complications and PLOS still exists in larger studies and high-quality studies.

### *Sensitivity analysis*

We aimed to investigate the influence of a single study on the overall results by omitting one study in each turn. This analysis revealed that no single study generated an

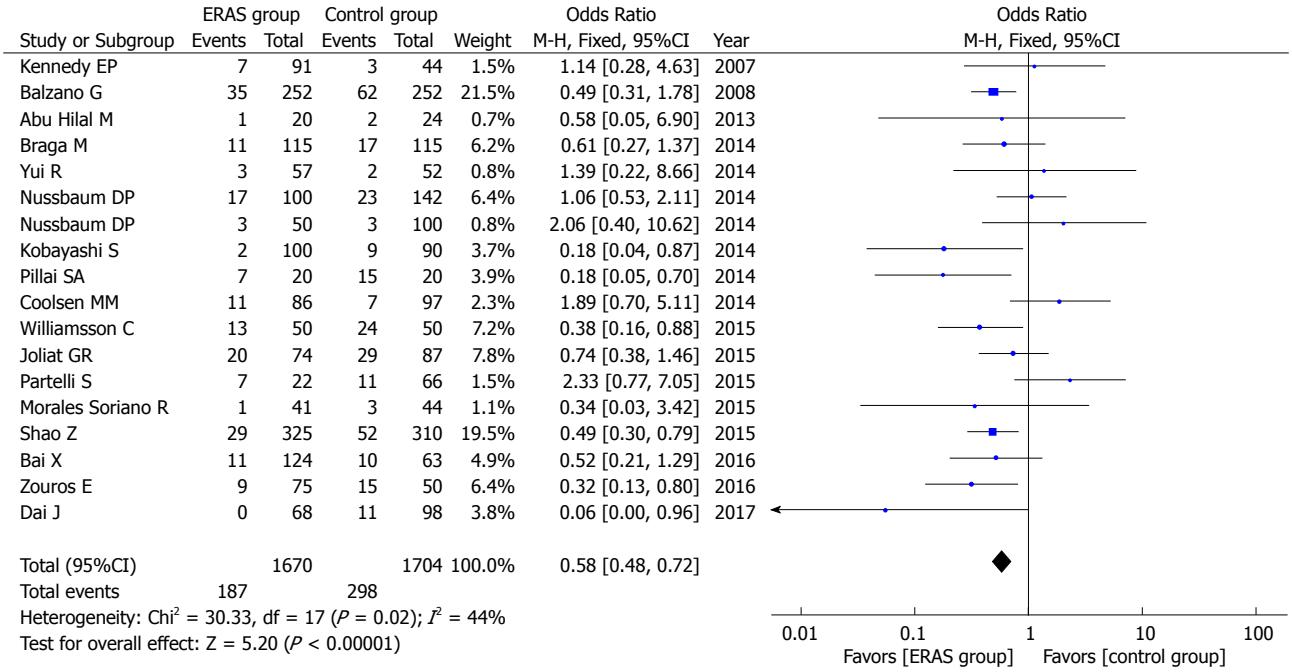


**Figure 3 Forest plots demonstrating the outcomes of delayed gastric emptying.**

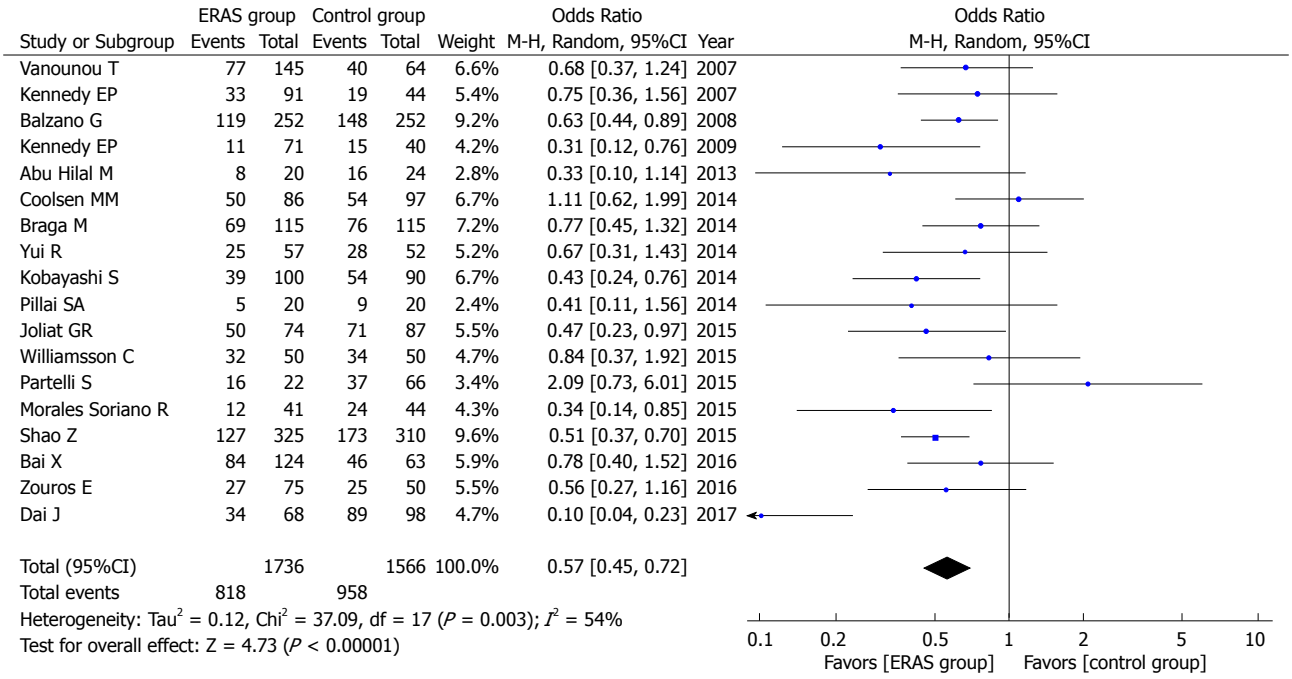


**Figure 4 Forest plots demonstrating the outcomes of overall complications.**

especially strong influence on the results, with estimates ranging from an OR of 0.54 to 0.62 (Table 4).

### *Publication bias*

Funnel plots based on the incidence of POPF and mortality were used to evaluate potential publication bias in this study (Figure 11). There was no evidence of publication bias of POPF, mortality or other outcomes of this study (other figures not shown).

## DISCUSSION

ERAS requires surgical, nursing, anesthesia, nutritionist and other specialties to work together and uses a series of optimal and evidence-based management measures to lessen perioperative surgical stress while promoting the recovery of organ function in the early postoperative period[34,35]. ERAS programs were initially implemented in colorectal surgery, with recommendations for each step

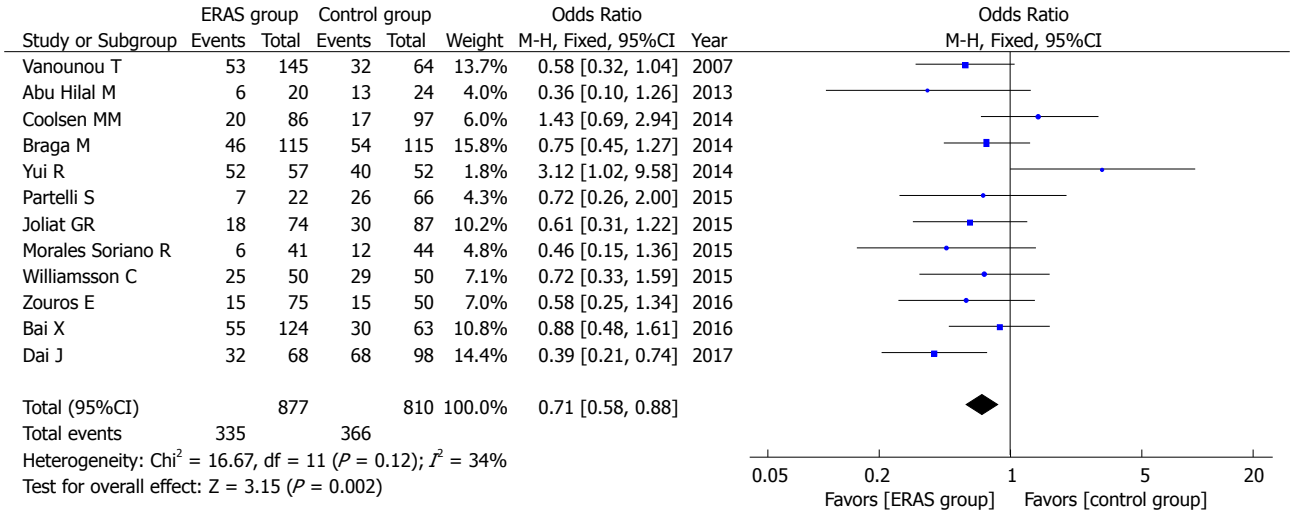


**Figure 5 Forest plots demonstrating the outcomes of mild complications.**

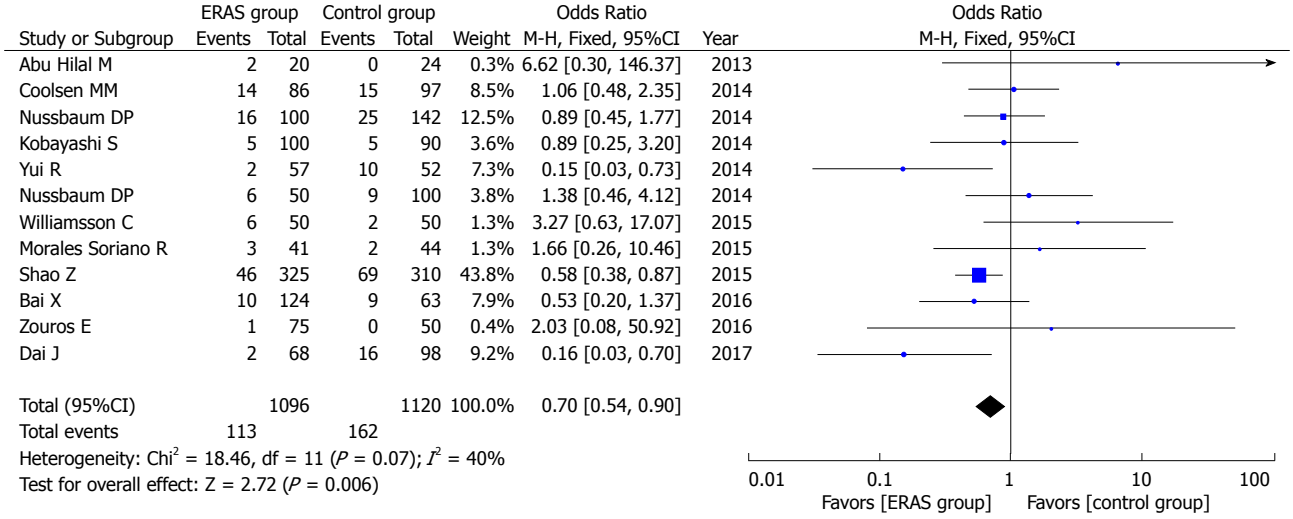


**Figure 6 Forest plots demonstrating the outcomes of abdominal infection.**

to achieve optimal perioperative care[36]. Subsequently, ERAS programs had been launched in numerous fields of surgery, such as orthopedics, urology, esophageal and gynecology.

The literature from these disciplines has suggested that standardizing ERAS measures could reduce the incidence of complications, accelerate recovery for patients, reduce hospitalization costs and save medical resources in perioperative care[3,4,7,8]. Pancreatic surgery is an effective treatment of pancreatic tumors, periampullary tumors, duodenal tumors and distal bile duct tumors. Currently, despite surgical techniques, anesthesia, and preoperative imaging assessment making great progress and the mortality of the procedure dropping to approximately 2% in high-volume medical centers, it is still considered a complicated and high-risk abdominal surgery[37].

Coolsen *et al*[38] analyzed 8 studies, which related to pancreatic surgery, and suggested that the ERAS group had shorter PLOS and lower postoperative complication rates; however, there were no significant differences in rates of DGE, POPF, readmission, and mortality. Kagedan *et al*[39] analyzed 10 studies suggesting that the ERAS group had only shorter PLOS and no differences in other complications. As mentioned above, we may reasonably conclude that the influence of ERAS programs on the postoperative complications of pancreatic surgery is controversial. Hence, the application of ERAS programs in the perioperative period of pancreatic surgery is still being explored in our practices.

The main measures of the ERAS programs include no bowel preparation and clear fluids until 2-3 h before surgery, multimodal analgesia of postoperative, clear fluids or food intakes, enhanced mobilization and removal of the drainage tube in early period**.** The ERAS group has reduced time of fasting in the preoperative period, which can decrease the insulin resistance in the postoperative period. We adopted multimodal

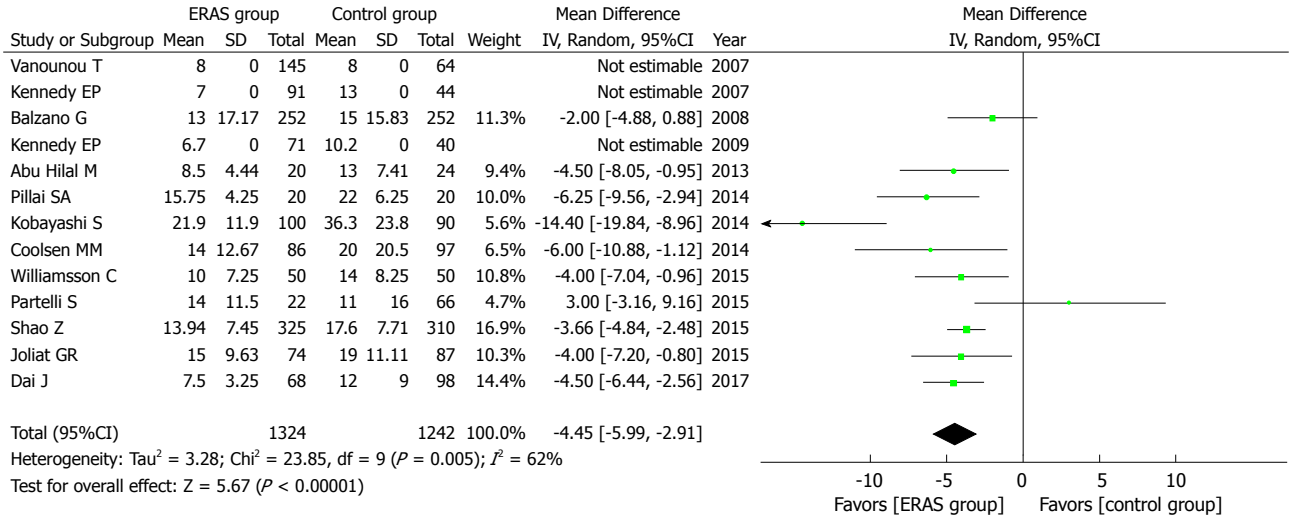


**Figure 7 Forest plots demonstrating the outcomes of postoperative length of hospital stay.**

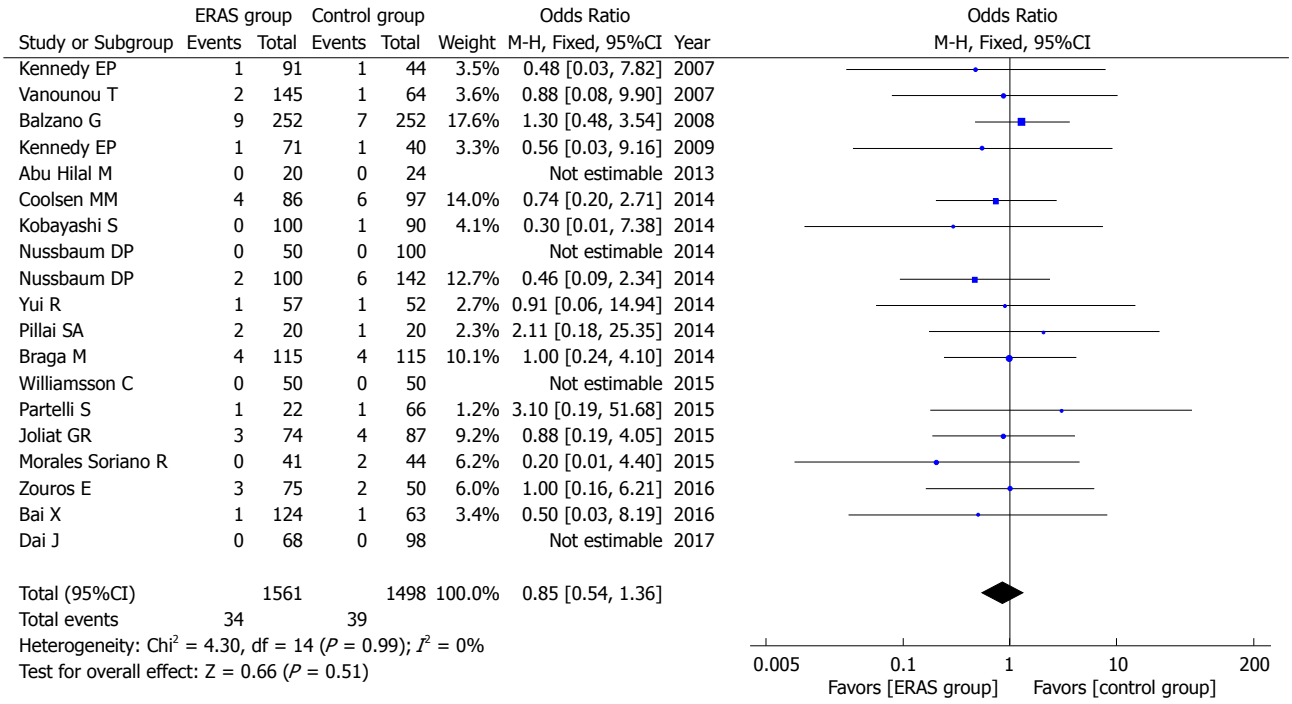


**Figure 8 Forest plots demonstrating the outcomes of mortality.**

analgesia in the postoperative period, which was able to reduce the stress caused by pain. The programs, such as, no bowel preparation before surgery, clear fluids or food intakes, enhanced mobilization in the early postoperative period which may promote rehabilitation of gastrointestinal function[40].

The ERAS programs aimed to reduce the incidence of complications and accelerate recovery for patients. Among them, gastrointestinal function rehabilitation is an important part of the rapid recovery in abdominal surgery. In addition, the early postoperative oral feeding, which may play an important role in the gastrointestinal function rehabilitation in the postoperative period. This is because early postoperative oral feeding is more in line with human physiology of the digestive tract, and which may have a beneficial effect on immunological, inflammatory and nutritional status. In addition, early postoperative oral feeding can promote the recovery of gastrointestinal motility, protect the gastrointestinal mucosal barrier, shorten time to gas and stools passage, and reduce the incidence of complications.

A total of 20 studies and 3694 patients were included in our meta-analysis. Compared with the control group, the ERAS group had lower rates of DGE, lower postoperative complication rates, particularly lower mild postoperative complication rates, lower abdominal infection rates, and shorter PLOS. However, no significant differences existed in POPF, moderate

| Study or Subgroup | ERAS group Events | Total | Control group Events | Total | Weight | Odds Ratio M-H, Fixed, 95%CI | Year |
|---|---|---|---|---|---|---|---|
| Kennedy EP | 7 | 91 | 3 | 44 | 2.5% | 1.14 [0.28, 4.63] | 2007 |
| Vanounou T | 13 | 145 | 4 | 64 | 3.4% | 1.48 [0.46, 4.72] | 2007 |
| Balzano G | 18 | 252 | 16 | 252 | 10.1% | 1.13 [0.56, 2.28] | 2008 |
| Kennedy EP | 5 | 71 | 10 | 40 | 8.1% | 0.23 [0.07, 0.72] | 2009 |
| Abu Hilal M | 1 | 20 | 2 | 24 | 1.2% | 0.58 [0.05, 6.90] | 2013 |
| Coolsen MM | 11 | 86 | 14 | 97 | 7.8% | 0.87 [0.37, 2.03] | 2014 |
| Braga M | 14 | 115 | 12 | 115 | 7.1% | 1.19 [0.52, 2.70] | 2014 |
| Nussbaum DP | 31 | 100 | 36 | 142 | 13.9% | 1.32 [0.75, 2.33] | 2014 |
| Nussbaum DP | 15 | 50 | 20 | 100 | 6.3% | 1.71 [0.79, 3.73] | 2014 |
| Pillai SA | 0 | 20 | 0 | 20 | | Not estimable | 2014 |
| Shao Z | 43 | 325 | 44 | 310 | 26.5% | 0.92 [0.59, 1.45] | 2015 |
| Partelli S | 3 | 22 | 11 | 66 | 3.2% | 0.79 [0.20, 3.14] | 2015 |
| Morales Soriano R | 4 | 41 | 4 | 44 | 2.4% | 1.08 [0.25, 4.64] | 2015 |
| Zouros E | 5 | 75 | 3 | 50 | 2.3% | 1.12 [0.26, 4.91] | 2016 |
| Bai X | 11 | 124 | 2 | 63 | 1.6% | 2.97 [0.64, 13.83] | 2016 |
| Dai J | 0 | 68 | 6 | 98 | 3.6% | 0.10 [0.01, 1.88] | 2017 |
| Total (95%CI) | | 1605 | | 1529 | 100.0% | 1.04 [0.83, 1.30] | |
| Total events | 181 | | 187 | | | | |

Heterogeneity: Chi$^2$ = 14.49, df = 14 ($P$ = 0.41); $I^2$ = 3%
Test for overall effect: Z = 0.31 ($P$ = 0.75)

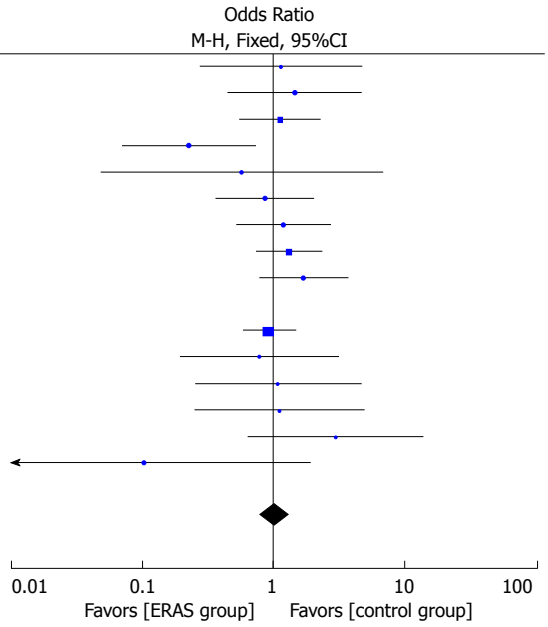


**Figure 9 Forest plots demonstrating the outcomes of readmission.**

| Study or Subgroup | ERAS group Events | Total | Control group Events | Total | Weight | Odds Ratio M-H, Fixed, 95%CI | Year |
|---|---|---|---|---|---|---|---|
| Vanounou T | 7 | 145 | 4 | 64 | 6.8% | 0.76 [0.21, 2.70] | 2007 |
| Balzano G | 17 | 252 | 20 | 252 | 24.1% | 0.84 [0.43, 1.64] | 2008 |
| Abu Hilal M | 0 | 20 | 3 | 24 | 4.0% | 0.15 [0.01, 3.08] | 2013 |
| Nussbaum DP | 10 | 100 | 18 | 142 | 17.3% | 0.77 [0.34, 1.74] | 2014 |
| Yui R | 1 | 57 | 1 | 52 | 1.3% | 0.91 [0.06, 14.94] | 2014 |
| Pillai SA | 3 | 20 | 1 | 20 | 1.1% | 3.35 [0.32, 35.36] | 2014 |
| Coolsen MM | 7 | 86 | 13 | 97 | 14.5% | 0.57 [0.22, 1.51] | 2014 |
| Braga M | 14 | 115 | 12 | 115 | 13.6% | 1.19 [0.52, 2.70] | 2014 |
| Morales Soriano R | 5 | 41 | 5 | 44 | 5.5% | 1.08 [0.29, 4.05] | 2015 |
| Partelli S | 1 | 22 | 3 | 66 | 1.9% | 1.00 [0.10, 10.14] | 2015 |
| Zouros E | 4 | 75 | 2 | 50 | 2.9% | 1.35 [0.24, 7.68] | 2016 |
| Bai X | 4 | 124 | 1 | 63 | 1.7% | 2.07 [0.23, 18.89] | 2016 |
| Dai J | 2 | 68 | 5 | 98 | 5.1% | 0.56 [0.11, 2.99] | 2017 |
| Total (95%CI) | | 1125 | | 1087 | 100.0% | 0.87 [0.63, 1.20] | |
| Total events | 75 | | 88 | | | | |

Heterogeneity: Chi$^2$ = 5.20, df = 12 ($P$ = 0.95); $I^2$ = 0%
Test for overall effect: Z = 0.84 ($P$ = 0.40)

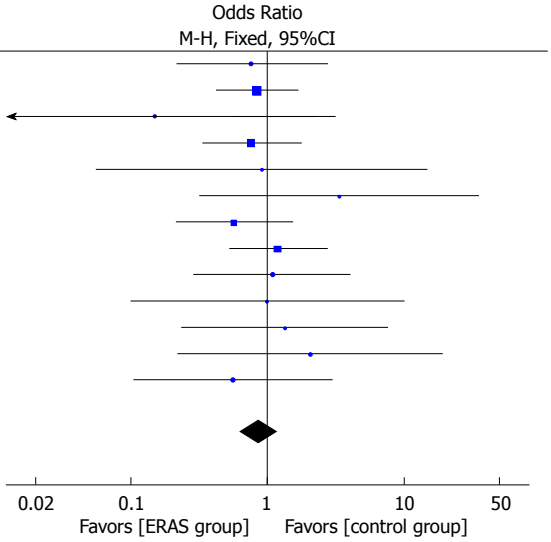


**Figure 10 Forest plots demonstrating the outcomes of unintended reoperation.**

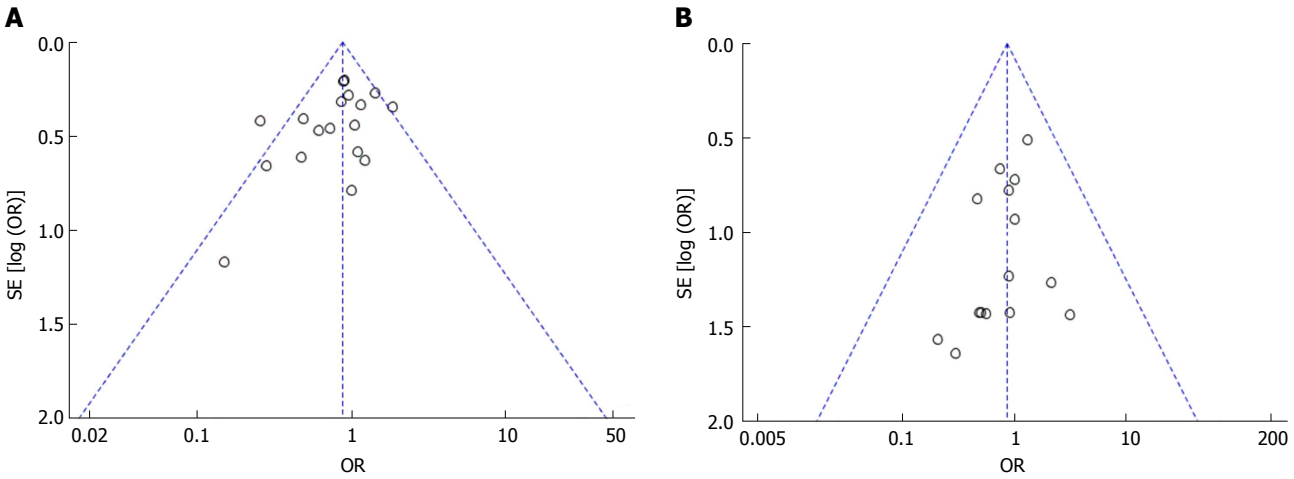


**Figure 11 The funnel plots were used to evaluate potential publication bias.** A: POPF; B: Mortality.

**Table 3 Results of Subgroup Analysis**

| Outcomes of interest | Studies | Patients | OR/WMD | 95%CI | *P*-value | Heterogeneity *P*-value | $I^2$, % |
|---|---|---|---|---|---|---|---|
| Studies with cases ⩾ 100 | | | | | | | |
| POPF | 14 | 3067 | 0.87 | 0.73-1.03 | 0.11 | 0.02 | 48 |
| DGE | 14 | 3117 | 0.58 | 0.47-0.71 | < 0.00001 | 0.07 | 39 |
| Overall complications | 14 | 3045 | 0.57 | 0.45-0.72 | < 0.00001 | 0.006 | 55 |
| Mild complications | 9 | 1470 | 0.74 | 0.59-0.93 | 0.009 | 0.06 | 46 |
| Abdominal infection | 10 | 2087 | 0.67 | 0.51-0.87 | 0.003 | 0.07 | 42 |
| PLOS | 10 | 2394 | -4.64 | -6.37 to -2.91 | < 0.00001 | 0.009 | 65 |
| Mortality | 15 | 2802 | 0.83 | 0.51-1.37 | 0.47 | 1 | 0 |
| Readmission | 12 | 2877 | 1.05 | 0.83-1.33 | 0.68 | 0.23 | 22 |
| Unintended reoperation | 9 | 1955 | 0.85 | 0.60-1.21 | 0.38 | 0.96 | 0 |
| MINORS score > 12 | | | | | | | |
| POPF | 15 | 2784 | 0.84 | 0.70-1.00 | 0.05 | 0.13 | 30 |
| DGE | 16 | 2949 | 0.52 | 0.42-0.64 | < 0.00001 | 0.12 | 31 |
| Overall complications | 16 | 3008 | 0.56 | 0.44-0.71 | < 0.00001 | 0.01 | 51 |
| Mild complications | 11 | 1504 | 0.67 | 0.54-0.83 | 0.0003 | 0.24 | 21 |
| Abdominal infection | 10 | 1791 | 0.63 | 0.46-0.85 | 0.002 | 0.05 | 46 |
| PLOS | 11 | 2272 | -4.35 | -5.97 to -2.72 | < 0.00001 | 0.003 | 66 |
| Mortality | 16 | 2523 | 0.96 | 0.56-1.65 | 0.89 | 0.99 | 0 |
| Readmission | 13 | 2598 | 1.09 | 0.84-1.43 | 0.52 | 0.82 | 0 |
| Unintended reoperation | 11 | 1787 | 0.96 | 0.65-1.41 | 0.83 | 0.94 | 0 |

CI: Confidence interval; DGE: Delayed gastric emptying; MINORS score: Methodological Index for Non-Randomized Studies checklist; OR: Odds ratio; PLOS: Postoperative length of hospital stay; POPF: Postoperative pancreatic fistula; WMD: Weighted mean difference.

**Table 4 Results of sensitivity analysis by omitting one study in each turn**

| Studies | OR | 95%CI | *P*-value |
|---|---|---|---|
| Omitting Vanounou *et al*[15] | 0.56 | 0.44-0.72 | < 0.00001 |
| Omitting Kennedy *et al*[14] | 0.56 | 0.44-0.71 | < 0.00001 |
| Omitting Balzano *et al*[16] | 0.56 | 0.43-0.73 | < 0.0001 |
| Omitting Kennedy *et al*[17] | 0.58 | 0.46-0.74 | < 0.00001 |
| Omitting Abu Hilal *et al*[18] | 0.58 | 0.45-0.73 | < 0.00001 |
| Omitting Yui *et al*[23] | 0.56 | 0.44-0.72 | < 0.00001 |
| Omitting Kobayashi *et al*[25] | 0.58 | 0.45-0.74 | < 0.00001 |
| Omitting Coolsen *et al*[21] | 0.54 | 0.43-0.69 | < 0.00001 |
| Omitting Braga *et al*[19] | 0.55 | 0.43-0.71 | < 0.00001 |
| Omitting Pillai *et al*[20] | 0.57 | 0.45-0.73 | < 0.00001 |
| Omitting Joliat *et al*[27] | 0.57 | 0.45-0.73 | < 0.0001 |
| Omitting Partelli *et al*[28] | 0.55 | 0.44-0.68 | < 0.00001 |
| Omitting Williamsson *et al*[29] | 0.56 | 0.44-0.71 | < 0.00001 |
| Omitting Morales Soriano *et al*[30] | 0.58 | 0.46-0.74 | < 0.00001 |
| Omitting Shao *et al*[26] | 0.57 | 0.44-0.74 | < 0.0001 |
| Omitting Zouros *et al*[32] | 0.57 | 0.44-0.73 | < 0.00001 |
| Omitting Bai *et al*[31] | 0.56 | 0.44-0.71 | < 0.00001 |
| Omitting Dai *et al*[33] | 0.62 | 0.52-0.71 | < 0.00001 |
| Overall effect | 0.57 | 0.45-0.72 | < 0.00001 |

CI: Confidence interval; OR: Odds ratio.

to severe complications, mortality, readmission or unintended reoperation in both groups.

Many factors, such as age, nutritional status, and serious comorbidity, can influence patients' postoperative complication rates and the process of postoperative recovery[41, 42]. The patients' demographic data in the included studies was basically identical, so these influences may be eliminated for the outcomes in this study. In addition, all of the included studies described the diagnostic criteria for postoperative complications.

Despite our careful work on this meta-analysis of currently available evidence, some limitations should be acknowledged. First, the diagnostic criteria of some postoperative complications were not uniformly defined, though all the included studies gave a description of the diagnostic criteria. Therefore, to a certain extent, information bias was possible, because some complications did not have national criteria. Second, only retrospective case control studies were included in this analysis. Therefore, to a certain extent, the outcomes of this study may be influenced by the selection bias. Third, the degree of implementation of ERAS programs and the compliance of patients may be different between studies. Finally, there was no evidence

to indicate that major publication bias existed in these studies, and potential publication bias is impossible to completely rule out in small studies. Hence, these factors had some influence on our results.

In summary, the results from our present study demonstrate that the implementation of ERAS programs could reduce overall complication rates, especially of mild complications, DGE, rates of abdominal infection, and PLOS, while not affecting the rates of POPF, reoperation, readmission, and mortality during the perioperative period for pancreatic surgery. The perioperative period for pancreatic surgery is safe and effective to implement ERAS programs that can decrease postoperative complication rates and promote recovery. However, in the future, we need to include more high-quality and strict prospective studies to assess the contributions of individual program components.

## ARTICLE HIGHLIGHTS

### Research background

Enhanced recovery after surgery (ERAS) is a multidisciplinary and evidence-based framework, developed to decrease perioperative surgical stress, accelerate postoperative recovery and significantly reduce the postoperative length of hospital stay (PLOS). ERAS programs have been launched in a variety of other fields of surgery, such as colorectal, orthopedics, urology, esophageal, and gynecology, and have demonstrated favorable outcomes. The implementation of ERAS programs has lagged surrounding pancreatic surgeries because of the anatomical location of the pancreas and the high rate of postoperative complications (30%-60%). It is very important to promote the postoperative recovery for this high-risk abdominal surgery via implementing ERAS programs during the perioperational period.

### Research motivation

ERAS requires surgical, nursing, anesthesia and other specialties to work together and uses a series of optimal or evidence-based management measures to lessen perioperative surgical stress while promoting the recovery of organ function in the early postoperative period. The implementation of ERAS programs may play a very important role in the perioperational period for pancreatic surgery.

### Research objectives

This study evaluated the impact of ERAS programs on postoperative complications and PLOS of pancreatic surgery.

### Research methods

Computer searches were performed in databases (including PubMed, Cochrane Library, and Embase) for randomized controlled trials or case-control studies describing ERAS programs in patients undergoing pancreatic surgery published between January 1995 and August 2017. Two researchers independently evaluated the quality of the studies' extracted data that met inclusion criteria and performed a meta-analysis using RevMan5.3.5 software. Forest plots, demonstrating the outcomes of the ERAS group *versus* the control group after pancreatic surgery, and funnel plots were used to evaluate potential publication bias.

### Research results

Twenty case-control studies, published between January 1995 and August 2017, including 3694 patients, were selected for the meta-analysis. They included the ERAS group ($n$ = 1886) and control group ($n$ = 1808), which adopted the traditional perioperative management. Compared to the control group, the ERAS group had lower delayed gastric emptying (DGE) rates (odds ratio (OR) = 0.58, 95% confidence interval (CI): 0.48-0.72, $P$ < 0.00001), lower postoperative complication rates (OR = 0.57, 95%CI: 0.45-0.72, $P$ < 0.00001), particularly for mild postoperative complications (Clavien-Dindo Ⅰ-Ⅱ) (OR = 0.71, 95%CI: 0.58-0.88, $P$ = 0.002), lower abdominal infection rates (OR = 0.70, 95%CI: 0.54-0.90, $P$ = 0.006) and shorter PLOS (weighted mean difference (WMD) = -4.45, 95%CI: -5.99 to -2.91, $P$ < 0.00001). However, there were no significant differences in postoperative pancreatic fistulas (POPF), moderate to severe complications (Clavien-Dindo Ⅲ-Ⅳ), mortality, readmission and unintended reoperation in both groups.

### Research conclusions

The results from our present study demonstrate that the implementation of ERAS programs could reduce overall complication rates, especially of mild complications, DGE, rate of abdominal infection and PLOS, while not affecting the rates of POPF, reoperation, readmission and mortality during the perioperative period for pancreatic surgery. The perioperative period for pancreatic surgery is safe and effective to implement ERAS programs that can decrease postoperative complication rates and promote recovery

### Research perspectives

We need to include more high-quality and strict prospective studies to assess the contributions of individual program components, such as clear fluids or food intakes in the early period, and removal of the drainage tube.

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**P- Reviewer**: Cesaretti M, Mastoraki A, Negoi I, Tang Y, Wani IA
**S- Editor**: Wang XJ **L- Editor**: Filipodia **E- Editor**: Huang Y

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ISSN 1007-9327