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Preventive health measures in inflammatory bowel disease

Ayokunle T Abegunde, Bashir H Muhammad, Tauseef Ali

Ayokunle T Abegunde, Tauseef Ali, Section of Digestive Diseases and Nutrition, Department of Medicine, Oklahoma University Health Sciences Center, Oklahoma City, OK 73104, United States

Bashir H Muhammad, Department of Medicine, Oklahoma University Health Sciences Center, Oklahoma City, OK 73104, United States

Author contributions: Abegunde AT, Bashir MH and Ali T contributed equally to the work; Ali T and Abegunde AT conceptualized and designed the review; Abegunde AT, Muhammad BH and Ali T contributed to search of literature and data extraction; Abegunde AT, Muhammad BH and Ali T drafted the manuscript; all authors reviewed and approved the final manuscript as submitted.

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Correspondence to: Tauseef Ali, MD, FACP, FACC, Section of Digestive Diseases and Nutrition, Department of Medicine, Oklahoma University Health Sciences Center, 920 Stanton L Young Boulevard, Oklahoma City, OK 73104, United States. tauseef-ali@ouhsc.edu
Telephone: +1-405-2715428

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Abstract

We aim to review the literature and provide guidance on preventive health measures in inflammatory bowel disease (IBD). Structured searches were performed in PubMed, MEDLINE, EMBASE, Web of Science and Cochrane Library from January 1976 to June 2016 using the following keywords: (inflammatory bowel disease OR Crohn's disease OR ulcerative colitis) AND (health maintenance OR preventive health OR health promotion). Abstracts of the articles selected from each of these multiple searches were reviewed, and those meeting the inclusion criteria (that is, providing data regarding preventive health or health maintenance in IBD patients) were recorded. Reference lists from the selected articles were manually reviewed to identify further relevant studies. Patients with IBD are at increased risk of developing adverse events related to the disease course, therapeutic interventions, or non-adherence to medication. Recent studies have suggested that IBD patients do not receive preventive services with the same thoroughness as patients with other chronic diseases. Preventive health measures can avert morbidity and improve the quality of life of patients with IBD. Gastroenterologists and primary care physicians (PCPs) should have an up to date working knowledge of preventive health measures for IBD patients. A holistic approach and better communication between gastroenterologists and PCPs with explicit clarification of roles will prevent duplication of services and streamline care.

Key words: Health maintenance; Prevention; Ulcerative colitis; Crohn's disease

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Core tip: Preventive health measures can avert morbidity and improve the quality of life of patients with inflammatory bowel disease (IBD). Gastroenterologists and primary care physicians (PCPs) should have an

up to date working knowledge of preventive health measures for IBD patients. A holistic approach and better communication between gastroenterologists and PCPs with explicit clarification of roles will prevent duplication of services and streamline care.

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INTRODUCTION

Inflammatory bowel disease (IBD) is one of the five most prevalent chronic gastrointestinal conditions in the United States, with an overall yearly health care cost of more than 1.7 billion^[1]. IBD has no cure and patients commonly require a lifetime of care; thus effective preventive measures to reduce morbidity, hospitalization, and surgery are critical to improving disease free remission and quality of life. Studies have shown that IBD patients do not receive preventive services with the same thoroughness as other medical patients^[2]. Several reasons for this disparity have been suggested; first, there is a greater focus on disease (symptom) control rather than preventive measures. Second, gastroenterologists are often the main care provider for patients with IBD and visits to the primary care physician (PCP) are often infrequent^[2,3]. Third, there is a lack of consensus regarding which provider (gastroenterologist or PCP) should offer preventive services and the limits of responsibility for providing preventive services^[2,3]. Other barriers to providing preventive services such as time constraints, patient refusal, and reimbursement issues have also been identified^[2,4]. These barriers to preventive care for IBD patients may ultimately affect the quality of care and patient outcomes. Therefore, gastroenterologists need to take a proactive role in the preventive health care needs of IBD patients by clarifying with each patient the limits of responsibility for preventive health and alerting PCPs to the unique health maintenance needs of IBD patients. Identifying which preventive measures are better delivered at the primary, secondary and tertiary care level is crucial to the delivery of quality care. This article reviews health interventions to prevent morbidity and mortality in IBD patients with emphasis on the location where such interventions are best delivered.

SEARCH STRATEGY

Bibliographical searches were performed in PubMed; MEDLINE; EMBASE; Web of Science and Cochrane Library from 1976 up to April 2016 using the following keywords: (inflammatory bowel disease OR Crohn's

disease OR ulcerative colitis) AND (health maintenance OR preventive health OR health promotion). Reference lists from the articles selected by electronic searching were manually reviewed to identify further relevant studies. Abstracts of the articles selected in each of these multiple searches were reviewed, and those meeting the inclusion criteria (that is, providing data regarding preventive health or health maintenance in IBD patients) were recorded.

SMOKING CESSATION

Smoking has a negative effect on patients with Crohn's disease (CD) in addition to the known risks of cardiac, respiratory, and oncologic disease. A survey of 675 IBD patients found that active smoking is a risk factor for CD and passive smoking is detrimental to the prognosis of CD. Among patients with UC, active smoking shows beneficial effects^[5]. Current smoking increases the risk of developing CD and worsens its course, increasing the need for steroids, immunosuppressive therapy (IST), and reoperations^[5]. On the contrary, smoking protects against UC, improves its course after disease onset, and decreases the need for colectomy^[5]. Therefore, achieving smoking cessation is an important goal of therapy in patients with CD. In patients with UC, the benefits of smoking on disease control must be balanced against the risk of non-IBD smoking related adverse effects. Smoking cessation is best achieved by a multidisciplinary approach through dedicated smoking cessation clinics with access to counselling, nicotine replacement therapy (NRT), and medication^[6,7]. Brief interventions during clinical encounters (asking patients about current smoking habit; advising them to stop smoking; assisting with NRT and arranging follow-up) may also be effective^[6,7]. Bupropion approximately doubles quit rates compared with placebo in the general population^[8]. In patients with CD, bupropion has been shown to be effective as an anti-smoking agent with mood stabilizing properties. The combination of NRT and bupropion was shown to improve the chance of achieving smoking cessation in CD patients by up to 20% compared with an unassisted quit attempt^[9]. NRT had no effect in modifying disease in UC patients who stopped smoking, suggesting that the beneficial effect of smoking in UC is not modulated *via* nicotine pathways. Varenicline is associated with higher rates of smoking cessation than bupropion and other forms of NRT^[8]. There are no RCTs using varenicline for smoking cessation in UC or CD; however, a cohort study (TABACROHN) has reported very good outcomes in CD patients treated with varenicline^[10]. Smoking cessation should be strongly advocated by gastroenterologists and PCPs in all CD patients that are current smokers because patients who quit smoking have a reduced number of relapses compared to continuing smokers [incidence rate ratio 0.84 (95%CI: 0.45-1.52) vs 1.53 (95%CI: 1.10-2.17)], which in turn reduces the use of biologics

Table 1 Pathogenesis of micronutrient deficiency in inflammatory bowel disease

Decreased food intake
Anorexia (TNF-mediated)
Mechanical (fistulas, post-operative)
Avoidance of high-residue food (can worsen abdominal pain/diarrhea)
Avoidance of lactose-containing foods (high rates of concomitant lactose intolerance)
Increased intestinal loss
Diarrhea (increased loss of Zn ²⁺ , K ⁺ , Mg ²⁺)
Occult/overt blood loss (iron deficiency)
Exudative enteropathy (protein loss, and decrease in albumin-binding proteins)
Steatorrhea (fat and fat-soluble vitamins)
Malabsorption
Loss of intestinal surface area from active inflammation, resection, bypass or fistula
Terminal ileal disease associated with deficiencies in B ₁₂ and fat-soluble vitamins
Hypermetabolic state
Alterations of resting energy expenditure
Drug interactions
Sulfasalazine and methotrexate inhibits folate absorption
Glucocorticoids impair Ca ²⁺ , Zn ²⁺ , and phosphorus absorption, vitamin C losses and vitamin D resistance
Cholestyramine impairs absorption of fat-soluble vitamins, vitamin B ₁₂ and iron
Long-term total parenteral nutrition
Can occur with any micronutrient not added to TPN
Reported deficiencies include thiamine, vitamin, and trace elements Zn ²⁺ , Cu ²⁺ , selenium, chromium

Adapted from Hwang *et al.*^[21]. TNF: Tumor necrosis factor.

and need for surgery^[10].

PHYSICAL ACTIVITY AND EXERCISE

IBD patients may have physical and psychological complaints that may impair their quality of life. Preliminary studies demonstrate that moderate exercise may diminish some symptoms of IBD^[11,12]. Additionally, the increasing prevalence of obesity in IBD patients may be associated with higher disease activity^[13,14]. Physical activity improves quality of life without detrimental effect on disease activity; it may also increase muscle mass and prevent osteoporosis^[14]. A retrospective cohort study of 240984 adolescent male military recruits revealed that physical fitness may reduce systemic inflammation levels relevant to the risk of symptomatic CD and UC^[12]. Low fitness was associated with an increased risk of IBD [unadjusted HR = 1.62 (95%CI: 1.31-2.00) for CD and 1.36 (95%CI: 1.17-1.59)] for UC^[12]. The inverse association of physical fitness with IBD risk suggests a protective role for exercise^[12]. However, the association between fitness and IBD may be due to prodromal disease activity reducing exercise capacity and therefore fitness^[12]. It has been hypothesized that the beneficial effect of regular exercise in IBD patients may be in part due to the anti-inflammatory effects of

myokines released during skeletal muscle contractions which inhibit the release of proinflammatory mediators from visceral fat^[13]. There is some evidence that PA may improve quality of life and reduce disease activity in patients with IBD^[15]. Thus PA may be useful as an adjunctive therapy in IBD by potentially improving psychological health, nutritional status, immunological response, bone mineral density and reversing the decrease of muscle mass and strength^[16,17]. Further studies are required to confirm these observations and establish exercise regimes for different IBD patient groups and an acceptable limit for physical activity in IBD patients.

NUTRITION

A diet rich in polyunsaturated fats and low in fiber may be associated with an increased risk of IBD^[18]. Enteral nutrition may improve CD flares and decrease the need for steroids in children and adolescents; however, no defined diets have been shown to consistently improve the disease course in adults with CD or UC^[19]. High intake of dietary fiber, particularly fruits and cruciferous vegetables is associated with decreased risk of CD, but not UC (HR = 0.59; 95%CI: 0.39-0.90)^[20]. Further studies are needed to define the role of specific diets in preventing disease progression in IBD.

Screening for malnutrition and micronutrient deficiencies

Patients with IBD are at increased risk of malnutrition via several mechanisms (Table 1)^[21]. IBD patients with clinical symptoms should be evaluated for their micronutrient status and identified deficiencies should be corrected. In IBD patients without clinical symptoms it is advisable to screen for common micronutrient deficiencies such as folate, iron and 25-hydroxyvitamin D^[21,22]. Patients with CD and extensive bowel resection are particularly at risk for vitamin B₁₂ deficiency, therefore, checking serum levels of vitamin B₁₂ is recommended^[21,22]. There are no current guidelines for assessment of micronutrient deficiencies in IBD patients and recommendations are based on expert opinion^[22].

SCREENING FOR ANEMIA

Anemia is one of the most common extra-intestinal manifestations of IBD^[23]. According to one study one-third of patients with IBD have hemoglobin levels below 12 g/dL^[24]. The anemic state is strongly correlated with quality of life, and is an important problem in the therapeutic management of patients with chronic disease^[25]. Anemia in IBD patients involves multiple pathogenic mechanisms. Although ongoing blood loss from chronically inflamed intestinal mucosa and micronutrient deficiency (iron and B₁₂)

are the main mechanisms underlying the development of anemia in patients with IBD, chronic inflammation, hemolysis, and medication-induced myelosuppression may also play important roles in both the development and management of anemia^[26,27]. Anemia of chronic disease and iron deficiency anemia are the two most common causes of anemia in patients with IBD^[28,29]. The World Health Organization defines anemia as hemoglobin levels < 13 g/dL (hematocrit < 39%) in males, < 12 g/dL (hematocrit < 36%) in non-pregnant females, and < 11 g/dL (hematocrit < 33%) in pregnant females^[30]. Severe IDA is defined as hemoglobin levels < 10 g/dL. If a patient meets WHO criteria for anemia, a basic anemia workup should be initiated to determine the cause of anemia. The basic workup includes a complete blood count, serum ferritin, transferrin, transferrin saturation, and C-reactive protein. If the cause of anemia is unclear despite the results of the above workup, more extensive testing is recommended. Further tests include vitamin B₁₂, folic acid, haptoglobin, lactate dehydrogenase, creatinine, and reticulocyte counts^[31]. Based on expert opinion and common clinical practice, screening for anemia in IBD patients is recommended at least every 3 mo for outpatients with active disease, and once every 6 to 12 mo for patients in remission or with mild disease; screening is not applicable to hospitalized patients^[31]. Treatment of anemia based upon the etiology should be started as soon as possible. In patients with IDA and hemoglobin > 10 g/dL, mildly active, or quiescent IBD, oral iron supplementation is adequate treatment^[32]. According to an international consensus statement, the preferred route of iron supplementation in IBD is intravenous, particularly, if the patient's Hgb level is < 10 g/dL in the setting of active disease^[31,33]. In a patient population with the predisposition for anemia, such as IBD patients, early diagnosis and management of iron deficiency can promptly reduce hospital visits, improve quality of life, reduce loss of work, and, ultimately, lower health care costs^[31,33].

BONE HEALTH

Prevention of osteoporosis and osteopenia

There is a high prevalence of osteoporosis (17%-41%) and osteopenia (22%-77%) in patients with IBD^[34,35]. Observational studies have shown a modest increased risk of osteoporotic fractures in patients with IBD compared to the general population^[34,35]. Dual-energy X-ray absorptiometry scanning is the gold-standard test for the diagnosis of osteoporosis and osteopenia^[36]. Osteoporosis is diagnosed when an individual's bone mineral density (BMD) at the hip or spine is more than 2.5 standard deviations below the mean for young healthy sex- and race-matched young adults (T-score less than -2.5). Osteopenia is diagnosed when the BMD is between -1 and -2.5 standard deviations (T-score of -1 to -2.5)^[36]. IBD patients are at increased

risk of fragility fractures in the absence low BMD for reasons that are not completely understood^[37]. The etiology of osteoporosis and osteopenia in IBD is multifactorial; risk factors include; corticosteroid use, age, malnutrition, vitamin D deficiency, calcium malabsorption, immobilization, degree of underlying GI inflammation and lower levels of sex hormones^[38]. Corticosteroid use is the strongest risk factor associated with osteoporosis. IBD patients on steroids for greater than three months have a significant increased risk of osteoporosis and fracture^[38]. Data on the prevention and treatment of osteoporosis in IBD are derived from observational studies and low quality RCTs in postmenopausal women with non-IBD inflammatory conditions^[39,40]. The American Gastroenterological Association guidelines recommend DEXA screening in IBD patients with one or more risk factors: history of vertebral fractures, postmenopausal females, males > 50 years of age, chronic corticosteroid therapy, or hypogonadism^[39]. If the initial DEXA is normal, the AGA recommends repeat testing in 2-3 years. If the patient has osteoporosis, or has a history of a low trauma fracture, evaluation for secondary causes should be completed. Suggested studies include a complete blood count, serum concentrations of alkaline phosphatase, calcium, creatinine, 25-OH vitamin D, serum protein electrophoresis, and testosterone level in males. Utilization of these guidelines potentially increases the number of screened patients and should lead to earlier diagnosis and treatment. In a single center cohort study of 100 consecutive IBD patients that met the AGA criteria for initial DEXA screening, osteoporosis was found in 12%, osteopenia in 44% and normal BMD in 44% of patients. Pharmacologic therapy was initiated in 89% of these patients, with 69% receiving calcium and vitamin D, and 20% receiving bisphosphonates^[41]. Although this was a small study with limited generalizability, it showed that following guidelines led to interventions that might ultimately reduce fracture risk^[41]. Interventions such as weight bearing, isotonic exercise, smoking cessation, avoiding alcohol excess, and maintaining adequate dietary calcium (> 1 g/d) have been shown to be beneficial in the prevention of osteoporosis in other inflammatory conditions and are recommended by the AGA^[39]. Minimizing the use of glucocorticoids is the most crucial intervention to prevent osteoporosis in patients with IBD. In patients with established osteoporosis, the use of bisphosphonates, calcitonin and its derivatives, raloxifene, and teriparatide have been shown to reduce or prevent further bone loss in post-menopausal women and men with hypogonadal osteoporosis^[42-44]. A recent meta-analysis showed that bisphosphonates are effective and well tolerated for the treatment of low BMD in male and female patients with IBD and reduces the risk of vertebral fractures^[43]. However there was insufficient data to support the efficacy of calcium and vitamin D, fluoride, calcitonin, or low-impact exercise in IBD patients^[43]. Hormone replacement therapy is no

Table 2 Prevention of osteoporosis in inflammatory bowel disease

Non-pharmacologic interventions
Regular weight-bearing exercise
Avoiding or quitting tobacco
Limited use of alcohol
Emphasis on better nutrition, particularly on vitamin D and calcium
Employment of fall prevention strategies
Pharmacologic interventions
Calcium and vitamin D supplementation
Bisphosphonates
Calcitonin
Cautious use of hormone replacement therapy for both women and men
Recombinant parathyroid hormone (teriparatide)
Minimizing corticosteroid use with the early use of immunomodulating agents

Adapted from Ali *et al*^[34].

longer recommended in post-menopausal women with IBD, given the increased risk of thrombosis (Table 2).

Calcium and vitamin D supplementation

Calcium (1200 mg/d) and vitamin D (400-800 IU/d) have been shown to be effective in fracture prevention in post-menopausal women^[45,46]. However, the evidence for calcium and vitamin D supplementation in the treatment of low BMD and prevention of fracture in IBD patients is limited^[47-49]. Calcium plus vitamin D supplementation resulted in a non-statistically significant improvement in lumbar and hip BMD^[47-49]. Further studies are needed and routine administration of calcium and vitamin D is not warranted in the absence of deficiency^[43].

EYE HEALTH

Approximately 10%-43% of patients with IBD develop eye problems^[50,51]. Ophthalmologic problems may be related to extraintestinal manifestations of IBD such as uveitis, episcleritis, or keratopathy or may be related to IBD therapy such as glucocorticoid-induced cataracts or glaucoma^[52,53]. Vitamin A deficiency may result in keratoconjunctivitis sicca after bowel resection in CD. Therefore it is recommended that patients with IBD undergo annual ophthalmologic evaluation, especially those on immunosuppressive therapy^[54].

SCREENING FOR IMPORTANT LATENT INFECTIONS

The majority of medications used to treat IBD are associated with immunosuppression which predisposes IBD patients to infection^[55]. Immunosuppression is defined as treatment with glucocorticoids (*e.g.*, prednisone 20 mg/d equivalent for 14 d or more), treatment with effective doses of 6-mercaptopurine (6-MP), Azathioprine (AZA), Methotrexate and anti-

tumor necrosis factor (Anti-TNF) therapy, either currently or within 3 mo of stopping therapy with any of the aforementioned medications, and significant protein-calorie malnutrition^[55]. Patients at high risk for TB infection such as those with prior exposure or patients with history of Bacillus Calmette-Guerin vaccine should be screened for latent or active TB prior to initiation of IST or biologic therapy^[55-57]. T-cell-based assays such as QuantiFERON (Cellestis) or T-Spot (Oxford Immunotec) are the preferred tests^[55-57]. Patients found to be positive for latent or active TB prior to initiation of IST should be referred to an infectious diseases (ID) specialist and treated before starting therapy. Similarly, patients who are found to be positive on IST should be referred to ID and treated after stopping IST^[55,56]. IBD patients should be screened for hepatitis B virus (HBV) prior to initiation of IST^[55-57]. Reactivation of HBV infection has been reported in IBD patients with chronic HBV (HBsAg positive) and prior exposure (HBcAb positive) on IST^[55-57]. IBD patients without prior exposure to HBV should be vaccinated and those with chronic infection should be treated prior to initiation of IST^[56,57]. There is no increased risk of reactivation of hepatitis C virus (HCV) with IST^[58]. Therefore screening for HCV should be performed on a case-by-case basis according to CDC guidelines^[59].

VACCINATION

Immunocompromised IBD patients are at a higher risk of infection by vaccine preventable diseases, therefore a diligent effort should be made to vaccinate all IBD patients. Ideally, these patients should be vaccinated before IST is initiated^[55,57]. Despite concerns for impaired immune response in immunocompromised IBD patients, most of these patients develop adequate response after vaccination^[55]. Live vaccines are contraindicated in immunocompromised IBD patients due to risks of vaccine associated infection; therefore the timing of live vaccines is particularly important when dealing with IBD patients on IST or those with plans to start IST^[55,57]. Special population groups such as pregnant patients, household contacts of immunocompromised patients, and travelers, pose special challenges^[55]. For example, a live vaccine administered to a household member of an immunocompromised IBD patient may expose the IBD patient to infection from the vaccinated family member. In general, it is recommended that household contacts of immunocompromised IBD patients be vaccinated according to recommended guidelines (Table 3). IBD patients embarking on foreign travel may warrant evaluation by an infectious disease specialist or travel medicine specialist^[55]. Table 4 provides general considerations for timing of live immunization in IBD patients and Table 5 provides general recommendations for vaccination in special populations (pregnant women and the IBD traveler)^[55].

Table 3 Vaccinations in inflammatory bowel disease summary (quick reference)

Vaccine	How often	Live Vaccine	Patients on Immunosuppressive therapy
Influenza (Flu vaccine)	1 dose every year	Nasal spray	Use flu shot only
Varicella (Chicken pox)	If no documented immunity: 2 doses, 4-8 wk apart	Yes	Contraindicated
Measles, mumps	If no documented immunity: 2 doses, 4 wk apart	Yes	Contraindicated
Rubella (MMR)			
Zoster (Shingles)	1 dose starting at 60 yr or older	Yes	Contraindicated
Tetanus, diphtheria, acellular pertussis (Td/Tdap)	If no prior vaccination: 3 doses (0, 1, 6-12). Then 1 dose of Tdap followed by a booster of Td every 10 yr	No	Follow recommended regimen
Human papilloma virus	Female: 3 doses through age 26 (0, 2 and 6 mo) Male: 3 doses through age 21 (0, 2 and 6 mo)	No	Follow recommended regimen
Pneumococcal (pneumonia vaccine) for subset of patients	If no prior vaccination: (0, 2 then 5 yr) 1 dose at 65 if had prior vaccination: 1 dose 5 yr after the last dose and 1 dose at age 65	No	Follow recommended regimen
Meningococcal (meningitis vaccine) for subset of patients	2 doses, 2 mo apart	No	Follow recommended regimen
Hepatitis A	2 doses, 6 mo apart	No	Follow recommended regimen
Hepatitis B	3 doses (0, 1 and 6 mo)	No	Follow recommended regimen

Centers for Disease Control and Prevention recommended vaccines for adults 2014, modified for inflammatory bowel disease patients.

Table 4 Live attenuated vaccines with recommended times of administration

Vaccine	Before initiation of immunosuppressive therapy	Already on immunosuppressive therapy
MMR	Contraindicated if starting therapy in 6 wk	Contraindicated
Zoster	Contraindicated if starting therapy in 4-12 wk	Contraindicated But could consider if: On short-term corticosteroids (< 14 d) On methotrexate (< 0.4 mg/kg per week) On azathioprine (< 3.0 mg/kg per day) On 6-mercaptopurine (< 1.5 mg/kg per day)
Varicella	Contraindicated if starting therapy in 4-12 wk	Contraindicated

Adapted from Chaudrey *et al*^[55].

SCREENING FOR SLEEP DISORDERS

Sleep disturbances have been strongly associated with IBD and other chronic inflammatory diseases such as Rheumatoid arthritis and lupus^[60,61]. Sleep disturbances in these diseases are due to cytokines produced by chronic inflammation. Increased levels of IL-1 and TNF- α are associated with an increase in non-rapid eye movement sleep^[61,62]. Nocturnal diarrhea disturbs sleep in IBD patients and the effects of slow wave sleep can lead to a decrease in colon contractility with direct effects on GI physiology such as diminished mucosal integrity^[63,64]. Studies have shown that IBD patients have poorer sleep quality, prolonged sleep latency, and increased use of sleeping pills compared with healthy controls^[64,65]. Furthermore, patients with clinically active IBD have significantly worse sleep than patients with inactive disease^[66-68]. IBD patients in clinical remission but with abnormal sleep are at increased risk of relapse at six months when compared to patients in clinical remission with good sleep^[67]. A cohort study of 3173 subjects showed that poor sleep increases the risk of relapse in patients with inactive CD but not UC^[68]. Sleep disorders can be a significant

quality of life issue and all patients with IBD should be screened and treated accordingly. In the future screening and treatment of sleep disorders might have therapeutic implications for management of IBD.

PSYCHOLOGICAL HEALTH

Psychological factors play an important role in IBD and have a wide range of impact on the social life of patients^[69]. The psychological well-being of patients is often affected by the disease's chronic relapsing nature, IST, and medication side effects^[69,70]. The presence of a psychological disorder in IBD is associated with poor health-related quality of life and self-perceived functional disability irrespective of symptom severity^[71,72]. As a result patients with psychosocial distress are less compliant and pursue greater healthcare utilization^[73,74]. Rates of depression in IBD range from 12.9%-27.2%^[73-77]. A population-based study found that the lifetime prevalence for major depressive disorder was more than twice as high in the IBD sample compared with controls (27.2% vs 12.3%, OR = 2.20, 95%CI: 1.64-2.95)^[77]. Although it is widely accepted that chronic diseases such as IBD

Table 5 Vaccination in special populations of inflammatory bowel disease patients

Category B	Pregnancy Category C	Category X
Influenza (LAIV)	PPSV 23	Varicella, if non-immune 1 dose upon completion or termination of pregnancy and before discharge from health care facility. 2 nd dose 4-8 wk later.
Influenza (IIV)	Zoster	
Boostrix (Tdap)	Adacel (Tdap)	
1 dose of Tdap vaccine during each pregnancy regardless of immunization status	1 dose of Tdap vaccine during each pregnancy regardless of immunization status	
HPV 4, HPV 2	Meningococcus	
PCV 13	Hepatitis A and B vaccine	
	MMR, if non-immune	
	1 dose upon completion or termination of pregnancy and before discharge from health care facility.	
	2 nd dose 4-8 wk later.	
	The IBD traveler	
Vaccine	Type	Travel related indication
Yellow fever	Live	Parts of South America and sub-Saharan Africa
Typhoid	Live and Inactivated	Asia, Africa, Central and South America, The Caribbean, Oceania
Polio	Live	
Influenza	Inactivated	
BCG vaccine	Live	Travel to highly endemic area > 1 yr
Hepatitis A	Inactivated	Central or South America, Mexico, Asia(except Japan), Africa, Eastern Europe
		sub-Saharan Africa, Saudi Arabia (during Hajj and Umrah pilgrimage)
Meningococcal vaccine	Inactivated	Rural Japan
Japanese encephalitis virus	Inactivated	

Adapted from Chaudrey *et al*^[55].**Table 6 Patient health questionnaire-2**

PHQ-2
Over the past month, have you felt down, depressed, or hopeless?
Over the past month, have you felt little interest or pleasure in doing things?

Adapted from Arroll *et al*^[78].

may trigger negative psychological emotions such as hopelessness and even depression, it is not clear if it is solely a psychological response to disease related morbidity, or whether it also represents a biological response of actual disease^[78,79]. Recently, a large cohort study showed that depression and anxiety were independent predictors of relapse in IBD^[76]. Therefore, special attention should be paid to screening IBD patients for depression on a regular basis by using simple screening questions such as the patient health questionnaire-2 (PHQ-2) (box 1)^[78,80] (Table 6). A positive answer to either of the screening questions mandates confirmatory testing with a standardized depression questionnaire such as the PHQ-9^[78,80]. A multidisciplinary approach involving the PCP, psychotherapist and psychiatrist is associated with the best outcomes in IBD and non-IBD patients^[75,80]. Patients with mild depression should be offered psychotherapy while patients with moderate to severe depression

should be offered psychotherapy and anti-depressant medication^[80,81]. In addition to controlling symptoms of anxiety and depression, anti-depressants such as selective serotonin reuptake inhibitors and serotonin norepinephrine reuptake inhibitors have been reported to decrease pain, gut irritability, and urgency of defecation^[75].

PREVENTION OF VENOUS THROMBOEMBOLISM

IBD patients have an increased risk of venous thromboembolism (VTE) due to the combined effects of inflammation, micronutrient deficiencies, hospitalizations, surgery and inherited prothrombotic factors^[82-84]. The relative risk (RR) of VTE in patients with IBD is inversely correlated with age; however, the actual incidence of VTE increases with age (45.6 per 10000 persons-year of follow-up)^[83]. IBD patients are 3.5 times more likely to develop VTE compared with age and gender matched controls in the general population^[83]. The risk of recurrence 5- years after discontinuation of anticoagulation therapy for an unprovoked VTE is higher among patients with IBD than controls (HR = 2.5, 95%CI: 1.4-4.2, $P = 0.001$)^[84]. VTE appears to carry a worse prognostic outcome for patients with IBD compared with the general population^[84,85]. In an analysis of a nationwide in-patient sample of

IBD patients in Canada, the rate of VTE was higher and the length of hospitalization was longer in IBD patients compared with controls (11.7 d vs 6.1 d, $P < 0.0001$)^[85]. Hospitalization was associated with higher costs (\$47515 vs \$21499, $P < 0.0001$) and higher mortality (adjusted OR = 2.5, 95%CI: 1.83-3.43) in IBD patients compared with controls^[85]. IBD patients undergoing surgery have an increased risk for post-operative VTE (OR = 1.26, 95%CI: 1.021-1.56, $P = 0.03$) compared with patients undergoing surgery for colorectal cancer^[86]. Therefore, it has been suggested that VTE prophylaxis in postoperative IBD patients be extended out-of-hospital for 4 wk after discharge^[87,88]. Given the increased risk of VTE in IBD patients, adequate prophylaxis should be prescribed for all IBD patients who are hospitalized with flares, or undergoing surgery^[87]. There are currently no clinical trials specifically addressing VTE prophylaxis and treatment in patients with IBD. However, consensus statements with specific recommendations were developed based on the 9th American College of Chest Physicians' guidelines on antithrombotic therapy and prevention of thrombosis with integration of evidence from IBD studies^[88]. Anticoagulant thromboprophylaxis is recommended for IBD patients who are hospitalized with IBD flares without active bleeding and when bleeding is non severe^[88]. Anticoagulant thromboprophylaxis is suggested during moderate severe IBD flares in outpatients with a history of VTE provoked by an IBD flare or an unprovoked VTE, but not otherwise^[88]. The recommended duration of anticoagulation after a first VTE is based on the presence of provoking factor^[87,88]. Please refer to the consensus document for details^[88].

CARDIOVASCULAR HEALTH

Hypertension

The increasing prevalence of obesity and metabolic syndrome in IBD patients increases the risk of primary essential hypertension^[89]. IBD patients are also at increased risk for secondary hypertension due to some of the medications used in treating the disease, such as corticosteroids and cyclosporine. Majority of drug induced secondary hypertension resolves after withdrawal of the offending medication. Lifestyle interventions with proven efficacy in non-IBD populations such as weight reduction, physical activity, reduction of dietary sodium, moderation of alcohol consumption are also effective in IBD-patients^[90-97]. If lifestyle interventions are not effective in lowering blood pressure, anti-hypertensive medication should be started in combination with lifestyle interventions per JNC 8 recommendations^[98].

Cerebrovascular accident

IBD is associated with increased risk of atrial fibrillation, stroke, myocardial infarction, hospitalization for heart

failure, and cardiovascular-related death during an IBD flare and during persistent disease activity, but not during remission^[99,100]. A meta-analysis of case-control and cohort studies showed that IBD is associated with a modest increase in the risk of CVA and ischemic heart disease, particularly in women^[101]. The reasons for the apparent increased risk in female patients with IBD compared with men is unclear, nevertheless, all patients with IBD should be counseled routinely on aggressive risk factor modification^[101].

Coronary artery disease

Patients with IBD have a modestly increased risk of coronary artery disease^[101,102]. Cardiovascular risk is higher in women with IBD and young adults (< 40-50 years) than in older adults (> 50-60 years)^[101]. Patients with IBD have evidence of premature vascular disease with structural, functional and biochemical changes indicative of subclinical atherosclerosis; IBD also promotes spontaneous platelet activation and aggregation, predisposing patients to arterial thrombosis^[102]. In a retrospective cohort study of 131 patients with IBD and CAD who underwent coronary angiography, it was observed that patients with IBD were less likely to have severe left anterior descending artery disease (56% vs 73%, $P < 0.01$) and multivessel disease (71% vs 79%, $P = 0.05$) than non-IBD controls with CAD (524 individuals)^[103].

IBD patients were also diagnosed with CAD at a younger age, had a lower body mass index, and were less likely to be active smokers^[103]. However, the results of that study should be interpreted with caution because the IBD- cohort consisted of patients with less severe CAD and low Framingham risk scores which may have biased the results towards more severe CAD in the non-IBD cohort. Though IBD disease activity (chronic inflammation) is directly related to the risk of cardiovascular events; the prevalence of traditional risk factors for CVD is not higher in IBD patients compared to the general population^[103,104]. IBD is associated with an increased risk of cardiovascular morbidity without increased cardiovascular mortality because chronic inflammation in the absence of traditional risk factors is not associated with an increased risk of premature CVD events^[105,106]. Screening for traditional risk factors for CVD such as HTN, diabetes, hyperlipidemia, family history, and smoking should be performed by PCPs or gastroenterologists. Non-traditional risk factors for CVD specific to IBD such as increased disease activity (elevated cytokines, CRP, ESR) should be identified by gastroenterologists^[102]. Identification of these risk factors should be followed by lifestyle interventions^[102]. Specific treatment should be provided by the PCP in conjunction with the cardiologist and gastroenterologist in a multidisciplinary strategy that involves aggressive use of disease-modifying biologic therapy to maintain remission in addition to the use of established evidenced based therapies such as aspirin,

statins, antihypertensives, and B-blockers^[107]. Non-invasive risk stratification should be performed when indicated to determine the need for escalation of therapy. Direct evidence from interventional studies on primary and secondary prevention of CVD events in IBD patients are lacking, therefore, the majority of recommendations are based on data from non-IBD populations^[107].

PREVENTING MEDICATION RELATED MORBIDITY

Corticosteroids

Steroids are very effective for inducing remission in IBD, but ineffective for maintaining remission. Long term use is associated with steroid related adverse effects; therefore early use of steroid sparing therapy is recommended to prevent steroid related adverse effects.

Non-steroidal anti-inflammatory drugs

High-dose non-steroidal anti-inflammatory drugs have been associated with an increase in disease activity in patients with CD or UC, while low-doses of NSAIDs were not associated with a higher disease activity index score among CD patients^[108]. Bonner *et al.*^[109] reported no association between NSAID use and increased disease activity in IBD, suggesting that NSAID use in IBD deserves further study before recommending that patients refrain from their use under all circumstances. Nonselective NSAIDs were associated with a 17%-28% relapse rate within 9 d of ingestion in patients with quiescent IBD^[110]. In another study, the adjusted odds ratio between NSAID use and relapse was 6.31 (95%CI: 1.16-34.38, $P = 0.03$)^[111]. Similarly, selective Cox-2 inhibitors have been associated with relapse in patients with CD and UC suggesting that all classes of NSAIDs are associated with relapse in IBD patients^[112,113]. However, other studies have reported no increase in flares and a beneficial safety profile with Cox-2 inhibitors during short-term treatment of IBD-associated arthritis and arthralgia^[114,115]. IBD patients should be advised to avoid NSAIDs; however some patients may require NSAIDs for management of arthritis. Therefore, a patient-centered approach involving discussions on the risks and benefits of NSAID use is warranted before considering NSAID use in IBD-patients.

Oral contraceptives

IBD often presents during the reproductive years; thus women with the disorder need effective contraceptives to prevent unintended pregnancies^[116].

Efficacy of oral contraceptive pill in IBD

Most absorption of oral contraceptive pill (OCP) steroids occurs in the small bowel^[117]. Factors that may impair absorption of OC sex steroids, such as

inflammation and ulceration of the intestinal mucosa, or more rapid transit of gastrointestinal contents in patients who have undergone bowel surgery, might reduce the efficacy of OCPs^[118]. Two pharmacokinetic studies compared the absorption of combined OCPs between women with UC and healthy volunteers^[119,120]. These studies found no significant differences in the absorption of OCPs among women with mild UC and those with an ileostomy following proctocolectomy with small ileal resections when compared with healthy women^[119,120]. It was also noted that women with the largest bowel resections and continent ileostomies had the absolute lowest plasma levels of 1-norgestrel, however the plasma levels never dropped below the threshold needed to inhibit ovulation^[120]. Thus PCPs may need to decide on a higher dose of OCPs for patients with extensive bowel resections for CD.

Effect of OCP on incidence and relapse in IBD

Multiple studies have examined the relationship between use of OCPs at or after a diagnosis of IBD and incidence of relapse, none of which found a significant effect of OCP use on a variety of measures of relapse^[121-127]. One study found a significantly increased risk of relapse (HR = 3.0; 95%CI: 1.5-5.9) among CD patients who were current or previous OC users when the reference group was never users (including men)^[123]. The increased risk was predominantly accounted for by the high rate of relapse among previous OC users (70%), which was higher than the relapse rate among current OC users (43%)^[123]. However, a comparison of current users alone with never users failed to find an increased risk of relapse among current users^[123].

Another prospective cohort study found that women who continued to take OCPs were at a threefold increased risk of developing a relapse of CD; the risk was higher among women who were prescribed OCPs and smoked, suggesting that smoking was a confounding variable^[125].

OCP and risk of thromboembolism in IBD

No studies were found that directly examined the risk of thrombosis among women with IBD who were using hormonal contraceptives. However, a prospective cohort study examining the risk of CD relapse among 331 women (134 oral contraceptive users and 197 nonusers) over a 12-mo period reported no cases of arterial or venous thrombosis^[122]. Kane *et al.*^[126] found hormone replacement therapy (HRT) to be protective against disease activity in post-menopausal women with IBD (HR = 0.18, 95%CI: 0.04-0.72). A dose-response effect was noted with longer duration of HRT; however the results should be interpreted with caution because it was a small single center retrospective study with limited generalizability^[126]. The evidence regarding other adverse health outcomes associated with contraceptive use among women with IBD is

limited to a single case report that discussed the possible relationship between use of combined OCPs and the development of ischemic colitis in a woman who had recently undergone surgery for obstructing CD^[127]. The balance of current evidence suggests that OCPs are associated with a reduced rate of relapse and can be used in women of childbearing age with IBD unless further large-scale studies prove otherwise. The same precautions when prescribing OCPs to women of child bearing age without IBD should be applied to women with IBD of child bearing age^[128].

Antibiotics

Antibiotic use can induce selection pressure and alter the gut microbiome^[129]. Different studies have shown that antibiotic use at different ages ranging from infancy to adulthood can increase the risk for IBD^[130,131]. In contrast, antibiotics have been shown to be protective in many large scale studies in patients with established IBD. In a meta-analysis of RCTs, antibiotics were superior to placebo at inducing remission in patients with active CD (RR of CD not in remission = 0.85; 95%CI: 0.73-0.99, $P = 0.03$)^[132]. Antibiotics were also superior to placebo for reducing fistula drainage in CD patients with perianal fistulae (RR = 0.8; 95%CI: 0.66-0.98)^[132]. In patients with active UC; antibiotics were superior to placebo for inducing remission (RR of UC not in remission = 0.64; 95%CI: 0.43-0.96)^[132]. Antibiotics were superior to placebo for inducing remission in patients with active CD and preventing relapse in patients with quiescent CD (RR of relapse = 0.62; 95%CI: 0.46-0.84)^[132]. The evidence on the effect of antibiotics on disease activity and relapse is limited because a diverse number of antibiotics with different spectra of activity were grouped together in the meta-analysis. Specifically, Nitroimidazoles (metronidazole and ornidazole) have been shown to be effective in preventing post-operative recurrence of CD^[133,134]. Although antibiotics are effective in treating mild flares of IBD and preventing post-operative recurrence in CD, caution is advised against indiscriminate use because of the risk of altering the composition of the gut microbiota which may increase the risk of a disease flare and *Clostridium difficile* infection.

Probiotics

The effect of probiotics on IBD is unclear, multiple studies using single agent probiotics have failed to show clinical benefit for disease control and prevention of recurrence^[135]. However, some studies have shown that VSL#3, a mixture of *Lactobacillus casei*, *L. plantarum*, *L. acidophilus*, *B. breve*, *B. infantis*, *Bifidobacterium longum* *L.delbrueckii* subsp. *bulgaricus*, and *Streptococcus salivarius* subsp. *thermophiles*, was able to induce remission in patients with mild-to-moderately active UC^[136]. In CD patients with a history of ileocolonic resection and reanastomosis there was no statistically

significant difference between VSL#3 and placebo in preventing post-surgical recurrence of CD^[137]. Based upon existing data the only proven benefit of probiotics was shown in the postoperative period in UC patients with ileal pouch-anal anastomosis (IPAA)^[138]. VSL#3 has been shown to maintain remission and prevent episodes of pouchitis in UC patients with IPAA^[138].

5-aminosalicylic acids

5-aminosalicylic acids use has been associated with an increased risk of renal disease and folate deficiency^[21,139]. Folate supplementation 1 mg/d and annual renal function monitoring is recommended for patients taking 5-Aminosalicylate medication^[21,139].

Thiopurines

Measurement of thiopurine methyltransferase (TPMT) activity is recommended prior to starting treatment with thiopurine drugs such as AZA and 6-MP. Approximately 10% and 0.3% of the general population will have low activity and absent activity respectively^[140,141]. Patients with low activity or absent activity are at an increased risk of drug-induced bone marrow toxicity and myelosuppression due to accumulation of the unmetabolized drug^[140,141]. Therefore, TPMT, CBC and liver function should be checked prior to initiating therapy and CBC and liver function should be monitored while on therapy^[140].

Methotrexate

Treatment with methotrexate suppresses the immune system and depletes folate. CBC, liver, and renal function should be checked prior to initiating therapy and monitored while on therapy. Patients should also receive daily folate supplementation 1 mg/d^[140].

Anti-TNF-alpha agents

Anti-TNF agents are associated with immunosuppression, increased risk of infections, and reactivation of latent infections - see section on infection. CBC, liver, and renal function should be checked prior to initiating therapy and periodically monitored while on therapy^[140].

Anti-integrin antagonists

Natalizumab a humanized monoclonal antibody against alpha-4 ($\alpha 4$) integrin has been associated with reactivation of the John Cunningham (JC) virus and cases of progressive multifocal leukoencephalopathy (PML)^[142]. The drug is only available through a restricted program (TOUCH Prescribing Program). It is recommended that anti-JCV antibody be checked prior to treating patients with Natalizumab. CBC and liver function should be monitored while on therapy, and patients should be monitored for any new sign or symptom suggestive of PML. Vedolizumab is a humanized monoclonal antibody against alpha-4 beta-7 ($\alpha 4\beta 7$) integrin which specifically targets mucosal addressin cell adhesion molecule (MAdCAM)

resulting in gut-selective anti-inflammatory activity^[143]. CBC, liver, and renal function should be checked prior to initiating therapy and monitored while on therapy.

CANCER SCREENING

Skin cancer

Several observational studies have reported an increased risk of melanoma skin cancer and non-melanoma skin cancer (NMSC) in patients with IBD^[144-147]. The risk of NMSC was shown to be higher in IBD patients on antimetabolites (AZA and 6-MP), anti-TNF agents, or combination therapy (anti-TNF + AZA or 6MP)^[147]. Patients should be advised to minimize exposure to UV radiation by wearing cover up clothing, UV-blocking sunglasses and a broad-brimmed hat when feasible, use broad spectrum (UVA/UVB) sunscreen with SPF of 15 or higher every day, and avoid tanning beds. For extended outdoor activity, patients should be advised to use of a water-resistant, broad spectrum (UVA/UVB) sunscreen with SPF of 30 or higher^[148]. Monthly skin self-examination and annual physician skin exam should be considered in patients on immunosuppressive therapy.

Cervical cancer

The incidence of abnormal Pap smears is higher in women with IBD compared to the general population, and the risk of abnormal Pap smears increases with treatment with IST^[149,150]. However, other studies suggest that concurrent IST and smoking may explain the association between IBD and cervical dysplasia rather than just the diagnosis of IBD^[151,152]. Kane^[149] reported a 42.5% incidence of abnormal Pap smears among women with IBD compared with 7% among controls matched for age, sex and parity. Interestingly, all abnormal tests were associated with either HPV serotype 16 or 18, which underscores the importance of screening female IBD patients for cervical cancer and HPV infection before, soon after initiating, or during IST^[149]. Females with IBD between the ages of 11 and 26 years on IST should be considered for the HPV vaccine^[55]. All women on IST should undergo annual Pap testing as recommended by the American College of Obstetrics and Gynecology's guidelines^[153].

Breast cancer

Breast cancer is the most common malignancy in United States women and the second leading cause of cancer related mortality among women in the United States^[154]. Modest evidence suggests that first-degree relatives, particularly, mothers' of CD patients may have a 2-fold higher rate of breast cancer compared with controls^[155]. Additional evidence suggests that CD patients with breast cancer are not treated as aggressively as controls and they have lower survival when treated, than patients without CD^[156]. The majority of IBD patients are classified as average risk

for breast cancer screening purposes and should be screened according to American Cancer Society (ACS) guidelines which recommend that average-risk women should receive counseling to raise awareness of breast cancer symptoms and a clinical breast examination (CBE) every 3 years starting at the age of 20 years, followed by annual mammography and CBE beginning at the age of 40 years^[154]. Female IBD-patients with high risk breast cancer genetic syndromes (*i.e.*, BRCA mutation), IBD patients with a first degree relative with a high-risk genetic syndrome or IBD patients with greater than 20% lifetime risk of breast cancer based on risk-estimation models should be considered high risk and screened according to ACS guidelines for high risk patients^[154].

Colorectal dysplasia and cancer

Patients with long-standing IBD are at an increased risk for developing colorectal dysplasia and cancer^[157,158]. According to the AGA guidelines, all patients, regardless of extent of disease, should undergo a screening colonoscopy^[158]. There is consensus that if a patient with UC or CD is found to have confirmed low-grade dysplasia in flat mucosa, proctocolectomy or repeat surveillance within 6-mo should be offered^[157,158]. Preventive surgery (proctocolectomy) is recommended in patients with high grade dysplasia. If a patient with extensive UC or CD involving the colon has had disease for 8 to 10 years, surveillance colonoscopy should be performed every 1 to 3 years^[157,158]. There is ongoing debate over the optimal number biopsies that should be obtained and the endoscopic method for CRC surveillance in IBD^[159,160]. However, a recent international consensus statement suggests that chromoendoscopy may be superior to white-light endoscopy for detection of dysplasia^[161]. This international consensus statement also favors endoscopic management over colectomy for the management of polypoid, non-polypoid and invisible dysplasia in IBD patients; however the recommendations are based on very low quality evidence^[161]. Please refer to the complete guidelines on the appropriate surveillance for colorectal cancer in IBD^[157,158].

Prostate cancer

Screening for prostate cancer is controversial because a significant proportion of cases detected by screening will not cause symptoms during the lifetime of affected patients^[162]. Consequently, many patients may not benefit from screening and may, be harmed by early cancer detection and treatment^[154,162]. Therefore, a discussion about the potential benefits and risks associated with testing should occur between the physician and patient before testing. The ACS guidelines emphasize the importance of shared decision-making between the physician and patient and recommends PSA testing for men aged 50 years who are at average risk of prostate cancer and are expected to live at least 10 more years^[154]. Patients at

Table 7 The 8-item Morisky Medication Adherence Scale

Morisky adherence scale question	Scoring
Do you sometimes forget to take your pills?	1 for NO; 0 for YES
People sometimes miss taking their medications for reasons other than forgetting. Thinking over the past 2 wk, were there any days when you did not take your medication?	1 for NO; 0 for YES
Have you ever cut-back or stopped taking your medication without telling your doctor, because you felt worse when you took it.	1 for NO; 0 for YES
When you travel or leave home do you sometimes forget to take your IBD medication?	1 for NO; 0 for YES
Did you take your IBD medicine yesterday?	1 for NO; 0 for YES
When you feel that your IBD symptoms are under control do you sometimes stop taking your medication?	1 for NO; 0 for YES
Taking medication every day is a real inconvenience for some people. Do you ever feel hassled about sticking to your IBD treatment plan?	1 for NO; 0 for YES
How often do you remember to take all your IBD medications?	
Rarely/Never	1.00
Once in a while	0.75
Sometimes	0.50
Usually	0.25
Always	0.00

Adapted from Trindade *et al.*^[167]. Scoring: < 6 points = low adherers; 6-7 points = medium adherers; 8 points = high adherers. IBD: Inflammatory bowel disease.

high risk of developing prostate cancer such as African Americans and men who have a first-degree relative (father, brother, or son) or more than one first-degree relative diagnosed with prostate cancer at an early age (< 65 years), the ACS recommends screening at 45 years and 40 years respectively^[154]. In contrast, the United States Preventive Services Task Force does not recommend PSA-screening because the benefits of PSA-based screening for prostate cancer do not outweigh the harms^[162].

SCREENING FOR MEDICATION NON-ADHERENCE

Non-adherence to IBD therapy leads to poorer disease outcomes^[163-165]. A recent case control study showed that about one-third of IBD patients were low adherers^[163], predictors of low adherence were age (< 40 years), higher educational level, being single, and mesalamine use^[163]. Interestingly, being self-employed was found to be a protective factor^[163]. Another study showed that non-adherence to anti-TNF agents was strongly associated with loss of response to anti-TNF agents^[165]. Longer intervals between outpatient clinic visits (≥ 3 mo) and limited knowledge of the prescribed medication were found to be significant predictors of non-adherence in that study^[165]. Taken together, limited knowledge about IBD medication and inappropriate self-perceptions of illness play a significant role in fostering non-adherence in IBD-patients, highlighting an unmet need for patient education and interventions aimed at improving adherence. Several strategies exist for monitoring medication adherence such as pill counts, medication refill rates, checking for drug metabolites and direct inquiry about medication adherence. Standardized questionnaires such as the 8-item Morisky Medication Adherence Scale (MMAS-8) may be used

to identify patients at risk of non-adherence with IBD therapy (Table 7)^[166]. The MMAS-8 has been criticized because it is a self-reported questionnaire which was validated on patients with chronic diseases that may not be entirely representative of IBD patients^[166]. Consequently, IBD specific screening tools to identify patients at risk of medication non-adherence such as the modified IBD-MMAS-8 and 10-item mesalamine non-adherence questionnaire were developed^[167,168]. There is modest evidence that the MMAS-8 reasonably identifies IBD patients at risk of non-adherence^[167,169]. Medication non-adherence can be categorized into two conceptual frameworks; accidental non-adherence and intentional non-adherence^[170]. On the basis of these categories, personalized algorithms may be developed to improve patient education, empowerment and follow-up. For example, strategies such as; patient-education, regimen simplification, use of reminder systems and organizational strategies (e.g., pill boxes) are likely to be best suited for addressing accidental nonadherence. In contrast, strategies such as teaching problem-solving skills, addressing motivational issues, and problematic patterns of family functioning are more likely to be effective in individuals displaying intentional non-adherence^[170].

CONCLUSION

Preventive health care can avert morbidity, mortality, and reduce overall health care costs^[171]. Patients with IBD require special healthcare needs at different stages of life from childhood through adolescence and adulthood. Historically, the role of the gastroenterologist was to achieve and maintain remission and monitor for adverse events. However, the potential risk of comorbidities negatively impacting IBD outcomes has mandated a working knowledge of health maintenance and a holistic approach to healthcare for IBD patients.

Table 8 Preventive health measures in inflammatory bowel disease

Morbidity	Preventive measures	Setting	Provider
Venous thromboembolism			
Dehydration	Encourage adequate hydration	Out-patient	PCP ¹ , Hospitalist ¹
	Intravenous fluids when indicated	In-patient	Gastroenterologist ²
Prolonged immobilization	Encourage physical activity	Out-patient	PCP ¹
	Early ambulation during hospitalization	In-patient	Hospitalist ¹
Indwelling catheters	Limit use of venous catheters when possible	In-patient	Hospitalist ¹ , Gastroenterologist ²
Hyperhomocysteinemia	Detection and correction of vitamin deficiencies B ₆ , B ₁₂ , folate	Out-patient	PCP ¹
			Gastroenterologist ²
Oral contraceptives	Advise on alternative methods of contraception	Out-patient	PCP ¹
Active intestinal disease (inflammatory burden)	Anti-inflammatory treatment, monitoring of medication and response to therapy.	Out-patient	Gastroenterologist ¹
			PCP ²
Cardiovascular disease			
Hypertension (Primary and secondary prevention)	Low sodium diet, smoking cessation, increased physical activity.	Out-patient	PCP ¹
	Anti-hypertensive medication		
Coronary artery disease (Primary and secondary prevention)	Low sodium diet, smoking cessation, increased physical activity, screening for hyperlipidemia. Statins, anti-platelet drugs,	Out-patient	PCP ¹
			Cardiologist ¹ , Gastroenterologist ²
Stroke (Primary and secondary prevention)	Anti-platelet therapy, statins, Anti-hypertensive medications	Out-patient	PCP ¹ , Neurologist ²
			Gastroenterologist ²
Smoking			
	Smoking cessation advise, nicotine replacement therapy, smoking cessation counselling and support programs	Out-patient	PCP ¹
			Gastroenterologist ²
Cancer			
Skin	Advise on UV exposure	Outpatient	PCP ¹
	Protective clothing, high SPF sunscreen		Gastroenterologist ²
	Yearly physician skin exam		
Colon	Surveillance colonoscopy per IBD guidelines	Out-patient	Gastroenterologist ¹
			PCP ²
Cervical	PAP smear	Out-patient	Gynecologist ¹ , PCP ²
Breast	Counselling on breast cancer awareness	Out-patient	PCP ¹
	CBE every 3 yr		
	Mammography after 40 yr		
Prostate	Counseling and Shared-decision making on PSA testing	Out-patient	PCP ¹
Nutritional deficiencies	Screen for and correct nutritional deficiencies	Out-patient	PCP ¹
			Gastroenterologist ²
Osteoporosis	DEXA in patients with increased risk of osteoporosis (hx of steroid use 10 mg daily x > 3 mo) treatment with bisphosphonates if osteoporosis confirmed.	Out-patient	PCP ¹
			Gastroenterologist ²
Infections			
Vaccine preventable infections	Vaccination	Out-patient	PCP ¹
			Gastroenterologist ²
Reactivation of Hepatitis B virus	Screening for HBV before initiating Anti-TNF therapy	Out-patient	Gastroenterologist ¹
Reactivation of latent Tuberculosis	Screening for latent TB before initiating Anti-TNF therapy	Out-patient	Gastroenterologist ¹
Anemia	Detection and treatment of anemia	Out-patient	PCP ¹
			Gastroenterologist ²
Depression	Depression screening PHQ 2	Out-patient	PCP ¹
	if positive do PHQ 9 for diagnosis		Gastroenterologist ²
	Mild depression -counselling		
	Moderate to severe- counselling +medication		
Sleep disturbance	Screening for sleep disturbance, Counseling on sleep hygiene	Out-patient	PCP ¹
			Gastroenterologist ²
	Medical therapy		
Medication related adverse effects	Assessing medication adverse effects and interactions	Out-patient	Gastroenterologist ¹
			PCP ²
Medication Non-adherence	Screening for medication non-adherence	Out-patient	Gastroenterologist ¹
	MMAS-8 item questionnaire		PCP ²
	Review frequency of medication refills		
	Drug levels for anti-TNF and thiopurines		

¹Primary role in preventive care; ²Secondary role in preventive care. PCP: Primary care physician; MMAS-8: 8-Morisky Medication Adherence Scale.

A recent study showed that incorporating a standard curriculum on IBD health maintenance provided fellows in training with increased awareness and guidance on managing the unique preventive care needs of patients with IBD^[172]. Different tools exist to assist practicing gastroenterologists in addressing preventive health issues during clinical encounters with IBD patients, such as, the Crohn's and Colitis Foundation of America (CCFA) check list for health maintenance or clinical assessment checklist developed by specialized IBD centers^[173-175]. The CCFA and AGA have publicized quality measures based on the best available evidence for processes and outcomes related to high quality care of IBD patients^[56,176]. Some recommendations in this review overlap with some of the CCFA and AGA quality measures for preventive health^[56,176]. Direct evidence from interventional studies in IBD patients are lacking for some preventive measures outlined in this review; therefore some recommendations are based on expert opinion and data from non-IBD populations. Additionally, this review considered the setting where each preventive intervention is best delivered (Table 8). Many of the interventions are appropriate for primary care settings and PCPs are uniquely qualified to provide interventions such as vaccination, smoking cessation, blood pressure control, referral for colon and cervical cancer screening^[177,178]. An educational intervention study providing instruction on diagnoses and management of IBD to PCPs in central Italy was associated a reduced rate of hospitalizations for IBD^[179]. Furthermore, a recent survey among gastroenterologists and PCPs in the United States reported that PCPs are very knowledgeable and comfortable providing primary care for IBD patients^[180]. Thus, the label "IBD" should not discourage PCPs from providing preventive health services to IBD patients. Evidence suggests that building therapeutic physician-patient relationships and shared decision-making is crucial to the outcome of chronic illnesses and IBD^[181,182]. Therefore, individual preference is likely to play a role in where and with whom patients choose to receive preventive health. Nevertheless, a holistic approach to care and better communication between gastroenterologists and PCPs with explicit clarification of roles will prevent duplication of services and streamline care.

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Challenges of advanced hepatocellular carcinoma

Stefano Colagrande, Andrea L Inghilesi, Sami Aburas, Gian G Taliani, Cosimo Nardi, Fabio Marra

Stefano Colagrande, Gian Giacomo Taliani, Cosimo Nardi, Dipartimento di Scienze Biomediche Sperimentali e Cliniche, University of Florence, I-50134 Florence, Italy

Andrea L Inghilesi, Sami Aburas, Fabio Marra, Dipartimento di Medicina Sperimentale e Clinica, Centro di Ricerca Denothe, University of Florence, I-50134 Florence, Italy

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Correspondence to: Stefano Colagrande, MD, Dipartimento di Scienze Biomediche, Sperimentali e Cliniche, Largo Brambilla 3, I-50134 Florence, Italy. stefano.colagrande@unifi.it
Telephone: +39-55-7947189
Fax: +39-55-431970

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malignancy, resulting as the third cause of death by cancer each year. The management of patients with HCC is complex, as both the tumour stage and any underlying liver disease must be considered conjointly. Although surveillance by imaging, clinical and biochemical parameters is routinely performed, a lot of patients suffering from cirrhosis have an advanced stage HCC at the first diagnosis. Advanced stage HCC includes heterogeneous groups of patients with different clinical condition and radiological features and sorafenib is the only approved treatment according to Barcelona Clinic Liver Cancer. Since the introduction of sorafenib in clinical practice, several phase III clinical trials have failed to demonstrate any superiority over sorafenib in the frontline setting. Locoregional therapies have also been tested as first line treatment, but their role in advanced HCC is still matter of debate. No single agent or combination therapies have been shown to impact outcomes after sorafenib failure. Therefore this review will focus on the range of experimental therapeutics for patients with advanced HCC and highlights the successes and failures of these treatments as well as areas for future development. Specifics such as dose limiting toxicity and safety profile in patients with liver dysfunction related to the underlying chronic liver disease should be considered when developing therapies in HCC. Finally, robust validated and reproducible surrogate end-points as well as predictive biomarkers should be defined in future randomized trials.

Key words: Barcelona Clinic Liver Cancer; Portal vein thrombosis; Modified Response Evaluation Criteria in Solid Tumors; Advanced hepatocellular carcinoma management; Advanced hepatocellular carcinoma second line therapies; Sorafenib

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Abstract

Hepatocellular carcinoma (HCC) is an aggressive

Core tip: Hepatocellular carcinoma (HCC) is an aggressive malignancy, which accounts for great part of all cancer deaths each year. Its management is com-

plex, and although the surveillance performed, many patients have an advanced stage. This comprehends an heterogeneous groups with different clinical condition; sorafenib is the only approved treatment, however affected by many adverse events. No single agent or combination therapies have been shown to impact outcomes after sorafenib failure. Loco-regional therapies as TAE/TACE and TARE have also been tested and at now are under evaluation. This review will focus on patients with advanced HCC and highlights potential and limit of the therapies.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the deadliest malignancies, ranking third as a cause of cancer death in males. Despite the recognition of cirrhosis as the major risk factor for HCC, more than 50% of patients with HCC present an advanced disease at diagnosis^[1]. Moreover, increased survival and better care for patients in earlier stages, allow their survival until they reach a more advanced stage.

The concept of "advanced" disease varies considerably analyzing the different staging systems utilized in the past ten years. One peculiarity of HCC is its association with chronic liver disease, especially cirrhosis. This makes prognosis of an individual patient dependent not only on the size, biologic behavior and spread of the tumor, but also on the degree of functional failure of the liver due to the presence of cirrhosis. The role of chronic liver disease in the prognosis of HCC is witnessed by the inclusion of the Child-Pugh score or other aspects linked to liver functions in several staging systems used for HCC (Table 1). In the Barcelona Clinic Liver Cancer (BCLC) staging system^[2], advanced HCC is considered as an unresectable HCC with/without extra-hepatic spread (metastases or lymph nodes involvement) and/or vascular invasion (portal or segmental invasion) and/or systemic symptoms, defined by an Eastern Cooperative Oncology Group performance status 1 or 2, with a liver function defined by a Child Pugh stage not greater than B^[3,4].

In this review we discuss several aspects of the management of patients with advanced HCC, focusing on the unmet needs that have emerged in the past few years, specifically since the introduction of sorafenib in clinical practice.

SORAFENIB IN THE TREATMENT OF ADVANCED HCC

The treatment of patients with advanced HCC has been for a long time disappointing for physicians. Curative options such as surgical resection or liver transplantation did not show any efficacy in prolonging overall survival (OS). Trans-arterial chemoembolization (TACE) in patients with advanced HCC due to portal vein thrombosis has been suggested to improve OS compared to patients receiving supportive care, in retrospective studies^[5] and in a recent meta-analysis, but is not currently recommended by practice guidelines^[6]. Early systemic therapies with hormone analogues (e.g., tamoxifen) or classic chemotherapeutic agents (e.g., doxorubicin) failed when tested in randomized controlled trials^[7]. In 2008 the approval of sorafenib in the until then desolated scenario of advanced HCC therapy radically changed the therapeutic approach, opening the era of molecular-targeted therapy. Till now, no additional molecules have yet been added to our pharmaceutical devices. Sorafenib is a multi-kinase inhibitor that suppresses tumor neo-angiogenesis and proliferation, inhibiting the tyrosine kinase activity of vascular endothelial growth factor receptors 1, 2 and 3 and of the platelet-derived growth factor receptor. It also inhibits the serine-threonine kinases Raf-1 and B-Raf^[8,9]. The efficacy of sorafenib has been demonstrated in two large independent randomized controlled trials. In the SHARP and Asia-Pacific studies the Authors reported an improvement in OS of almost 3 mo between the sorafenib and placebo arms (10.7 mo vs 7.9 mo and 6.5 mo vs 4.2 mo, respectively)^[10,11]. These results led to the approval of sorafenib for the treatment of advanced HCC. According to the technical schedule, the drug should be administered orally 400 mg b.i.d. until radiological progression or unacceptable adverse events occur.

Therapy is currently recommended in patients with preserved liver function, defined by a Child-Pugh score not greater than A, due to the exclusion of patients with more compromised liver function from randomized controlled trials. This represents a first major problem for sorafenib administration, as only a portion of patients can actually be treated. From the time of sorafenib approval, many field-practice studies have tried to evaluate the efficacy and tolerability of sorafenib in Child B patients, with conflicting results. The GIDEON study is so far the only prospective study that evaluated the impact of liver function in a large cohort of patients (> 3000), with a robust portion of subjects in Child-Pugh B class (666 patients)^[12]. In the final analysis, overall adverse events were similarly observed in both Child A and B patients, but

Table 1 Variables included in the most widely used hepatocellular carcinoma staging systems

Staging system	Ascites	Tumor burden	Albumin	Bilirubin	INR	HE	AFP	PVT	EHS	PS	ALP
Okuda	Yes	Yes	Yes	Yes	No	No	No	No	No	No	No
CLIP	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No
BCLC	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	No
GRETCH	No	No	No	Yes	No	No	Yes	Yes	No	Yes	Yes
TNM 7 th edition	No	Yes	No	No	No	No	No	Yes	Yes	No	No

AFP: Alpha fetoprotein; ALP: Alkaline phosphatase; EHS: Extrahepatic spread; HE: Hepatic encephalopathy; INR: International normalized ratio; PS: Performance status; PVT: Portal vein thrombosis.

a significant increase in serious adverse events was found in the Child B group. Moreover, Child-Pugh score was confirmed as a strong independent predictor of OS (5.2 mo in Child B vs 13.6 mo in Child A). The Authors concluded that sorafenib at full dosage is safe irrespective of the liver function. However, the use of full-dose sorafenib in a Child B patient is still far to be included in the clinical practice, as many physicians fear that the patients are too fragile in this subgroup. Additional trials specifically addressing this issue are ongoing (Sorafenib in First-line treatment of Advanced B Child Hepatocellular Carcinoma, clinicaltrial.gov).

An approach popular in the Hepatology community and potentially applicable to Child B patients is to start sorafenib at lower dosage (*e.g.*, 400 mg/d), ramping up to 800 mg/d in case of good tolerability. In case of poor tolerability, sorafenib should be continued at lower dosage, since data reported from the SOFIA group in 2011 did not show a reduction in OS in patients receiving half-dose sorafenib, whereas they actually had a significant survival advantage with respect to the group receiving full-dose sorafenib^[13]. Another rationale for the implementation of a ramp-up strategy could be the lower tolerability profile of sorafenib that seems to emerge from clinical practice. According to those studies, some of the most common adverse events (fatigue, diarrhea, hand-foot syndrome, bleeding, arterial hypertension, elevation of aminotransferase and/or bilirubin) are observed more frequently, in terms of incidence and severity, than reported in the registration trials. This leads to take into consideration a primary issue in sorafenib therapy, *i.e.* that an appropriate quality of life represents an essential goal in a non-curative treatment.

Another hot issue that has emerged from the recent literature is linked to the wide variability in survival and time to progression (TTP) observed in clinical practice. It is a general opinion that sorafenib therapy may be truly effective in a subgroup of patients, while it shows no real benefit in others. Identifying early predictors of response represents therefore a crucial research area, that becomes even more important if we consider the economic burden of the therapy^[14]. Numerous studies have explored the role of biochemical markers as prognostic factors or predictors of response. The concentrations of

alpha-fetoprotein, alkaline phosphatase, angiopoietin 2, Vascular Endothelial Growth Factor have been linked to improved survival, while soluble c-Kit and Hepatocyte Growth Factor have been proposed as predictive markers in field practice studies^[15-17] and in the SHARP trial. Observational studies have also linked the early development of adverse events like arterial hypertension, diarrhea or the hand-foot syndrome to a better response^[18-23]. Finally, clinical features such as the presence of macrovascular invasion have been associated with a worse prognosis^[21]. However, despite the large numbers of studies and the interesting results, no predictors have reached enough strength to be commonly used in clinical practice, due to the small sample size of most studies or to the lack of external validation of the findings. Therefore, although the aim of tailoring sorafenib therapy still appears exciting, tangible progresses will not be obtained without validation of parameters in large studies. Radiologic parameters also may represent an important tool in the management of sorafenib therapy.

As the majority of HCC develops in patients with chronic liver disease, treatment of the underlying condition and especially management of its complications, is mandatory. HBV infection accounts for about 60% of the total liver cancer in developing countries and for about 23% in developed countries^[24,25]. The benefits of antiviral nucleot(s)ide analogue therapy in improving recurrence-free survival and OS after curative treatment of HCC^[26] may suggest a possible role in improving outcomes also in advanced HCC, but at this time data on this topic are lacking.

BEYOND SORAFENIB: OTHER PHARMACOLOGIC APPROACHES TO THE MANAGEMENT OF ADVANCED HCC

The discovery of alternative lines of treatment for advanced HCC is an urgent unmet need. Sorafenib therapy is very expensive, and healthcare costs have become one of the main problems confronting governments and patients worldwide^[27]. Thus, in countries with limited health resources and a high incidence of HCC, a cost-effectiveness analysis to show the overall advantages of sorafenib is necessary. A

Table 2 Results of studies with molecular targeted therapies as first line in advanced hepatocellular carcinoma

Treatment	Trial	OS	TTP	Ref.
Sorafenib	Phase III <i>vs</i> placebo (SHARP)	10.7 mo <i>vs</i> 7.9 mo, $P < 0.001$; HR = 0.69; 95%CI: 0.55-0.87	5.5 mo <i>vs</i> 2.8 mo, $P < 0.001$	[10]
Sorafenib	Phase III <i>vs</i> placebo (Asia-Pacific)	6.5 mo <i>vs</i> 4.2 mo, $P = 0.014$; HR = 0.68; 95%CI: 0.50-0.93	2.8 mo <i>vs</i> 1.4 mo, $P = 0.0005$; HR = 0.57; 95%CI: 0.42-0.79	[11]
Sunitinib	Phase III <i>vs</i> sorafenib (SUN)	7.9 mo <i>vs</i> 10.2 mo, $P = 0.0019$; HR = 1.30; 95%CI: 1.13-1.50	4.1 mo <i>vs</i> 3.8 mo, one-sided $P = 0.8312$; two-sided $P = 0.3082$; HR = 1.13	[31]
Brivanib	Phase III <i>vs</i> sorafenib (BRISK-FL)	9.5 mo <i>vs</i> 9.9 mo, $P = 0.3116$; HR = 1.07; 95%CI: 0.94-1.23	4.2 mo <i>vs</i> 4.1 mo, $P = 0.853$; HR = 1.01; 95%CI: 0.88-1.16	[32]
Linifanib	Phase III <i>vs</i> sorafenib	9.1 mo <i>vs</i> 9.8 mo, $P = \text{NS}$; HR = 1.05; 95%CI: 0.90-1.22	5.4 mo <i>vs</i> 4.0 mo, $P = 0.001$; HR = 0.759; 95%CI: 0.64-0.895	[33]
Erlotinib	Phase III erlotinib plus sorafenib and sorafenib plus placebo (SEARCH)	9.5 mo <i>vs</i> 8.5 mo, $P = 0.408$; HR = 0.929	3.2 mo <i>vs</i> 4.0 mo, $P = \text{NS}$; HR = 1.135; $P = 0.18$	[36]

OS: Overall survival; PFS: Progression-free survival; TTP: Time to progression; CI: Confidence interval; HR: Hazard ratio; NS: Not significant.

Chinese study showed that the total cost was \$897 for patients in the best supportive care (BSC) group, while in the sorafenib group, the total cost was \$19495^[27]. Second, sorafenib is often discontinued for patients in whom the disease is progressed after sorafenib treatment^[28]. Many compounds and combinations have been explored in phase II or even phase III studies. Nevertheless, none of these have proven to be more effective than sorafenib as first-line therapy^[29,30] nor to be superior to placebo in second-line studies.

First-line treatments

The results of the SHARP trials have been a milestone opening the way to systemic therapy in advanced HCC. Nonetheless, the limited results in terms of survival benefit over placebo indicate that more effective first-line treatments are needed (Table 2). In the phase III SUN trial, sunitinib, a multi-kinase inhibitor inhibiting all vascular endothelial growth factor and platelet-derived growth factor receptors, was compared to sorafenib (400 mg) in patients with advanced HCC and the median OS was significantly shorter in the sunitinib arm (7.9 mo *vs* 10.2 mo) while TTP was not significantly different (4.1 mo *vs* 3.8 mo with sunitinib and sorafenib, respectively)^[31]. Of note, sunitinib was associated with severe adverse events, especially bleeding. The trial was prematurely discontinued for futility and safety reasons^[31].

Brivanib is a dual inhibitor of Vascular Endothelial Growth Factor and fibroblast growth factor receptors. A randomized phase III clinical trial has been conducted to evaluate the role of this drug as first-line therapy. The BRISK-FL study compared brivanib with sorafenib in patients with advanced HCC. This trial failed to meet the primary endpoint of improving OS (with 9.5 mo for brivanib and 9.9 mo for sorafenib) or other endpoints, including objective response rate, TTP (4.2 mo *vs* 4.1 mo) or disease control rates^[32].

Linifanib is another multi-targeted tyrosine kinase inhibitor, which has been evaluated as first-line therapy in comparison to sorafenib. Linifanib inhibits members of the Vascular Endothelial Growth Factor and Platelet-

derived growth factor receptors families. In the LIGHT phase III trial, linifanib was compared to sorafenib for efficacy and tolerability in patients with advanced HCC without prior systemic therapy. However, median OS was 9.1 mo on the linifanib arm and 9.8 mo on the sorafenib arm^[33], although TTP with linifanib was prolonged as compared with sorafenib (5.4 mo *vs* 4.0 mo, $P = 0.001$). Therefore, this trial failed to meet its primary endpoint and safety results favored sorafenib, as grade 3/4 or serious adverse events leading to discontinuation, dose interruption or reduction were more frequent with linifanib^[33].

Erlotinib is an orally active, potent and selective inhibitor of the human epidermal growth factor receptor, and its gene amplification has been reported in HCC^[34], although recent large scale results indicate that this occurs in a limited number of cases^[35]. This drug was tested in a phase III trial, where the efficacy and safety of a first-line treatment with sorafenib and placebo *vs* the combination sorafenib/erlotinib was evaluated in patients with advanced HCC^[36]. This trial failed to meet its primary endpoint, *i.e.*, an improvement in OS, the median values of which were 9.5 mo in the sorafenib plus erlotinib arm *vs* 8.5 mo in the sorafenib plus placebo group. Moreover, the median TTP (3.2 mo *vs* 4.0 mo) was not significantly different between the two arms^[36]. Withdrawal rates for adverse events were higher in the sorafenib/erlotinib arm. With regard to the drugs combination, a randomized phase II trial conducted in Child-Pugh A patients, comparing doxorubicin plus sorafenib or doxorubicin alone, combination therapy led to a longer median TTP (6.4 mo *vs* 2.8 mo, $P = 0.02$), OS (13.7 mo *vs* 6.5 mo, $P = 0.006$) and progression-free survival (6.0 mo *vs* 2.7 mo, $P = 0.006$) were observed^[37].

The results of a phase III study comparing sorafenib alone *vs* sorafenib plus doxorubicin have been recently presented in abstract form^[38]. The addition of doxorubicin to sorafenib resulted in higher toxicity and did not improve OS or progression-free survival. In another phase II study, first-line combination therapy with sorafenib and gemcitabine/oxaliplatin did

Table 3 Results of studies with molecular targeted therapies as second line in advanced hepatocellular carcinoma

Treatment	Trial	OS	TTP/PFS	Ref.
Brivanib	Brivanib <i>vs</i> placebo (BRISK-PS)	9.4 mo <i>vs</i> 8.2 mo, $P = 0.3307$; HR = 0.89; 95%CI: 0.69-1.15	4.2 mo <i>vs</i> 2.7 mo, $P < 0.001$; HR = 0.56; 95%CI: 0.42-0.76	[42]
Everolimus	Everolimus <i>vs</i> placebo (EVOLVE-1)	7.6 mo <i>vs</i> 7.3 mo, $P = 0.68$; HR = 1.05; 95%CI: 0.86-1.27	3.0 mo <i>vs</i> 2.6 mo, $P = 0.01$; HR = 0.93; 95%CI: 0.75-1.15	[44]
Ramucirumab	Ramucirumab <i>vs</i> placebo (REACH)	9.2 mo <i>vs</i> 7.6 mo, $P = 0.14$; HR = 0.87; 95%CI: 0.72-1.05	2.8 mo <i>vs</i> 2.1 mo, $P < 0.0001$; HR = 0.63; 95%CI: 0.52-0.75	[45]

OS: Overall survival; PFS: Progression-free survival; TTP: Time to progression; HR: Hazard ratio.

Table 4 Principal ongoing studies in advanced hepatocellular carcinoma with new molecular targeted therapies

Study	Drug	Status
A multicenter, open-label, phase 3 trial to compare the efficacy and safety of lenvatinib (e7080) <i>vs</i> sorafenib in first-line treatment of subjects with unresectable hepatocellular carcinoma	Lenvatinib <i>vs</i> sorafenib	Active, not recruiting
Study of regorafenib after sorafenib in patients with hepatocellular carcinoma (RESORCE)	Regorafenib <i>vs</i> placebo	Recruiting
A study of dovitinib <i>vs</i> sorafenib in adult patients with hepatocellular carcinoma as a first line treatment	Dovitinib <i>vs</i> sorafenib	Completed (phase 2)
A study of nivolumab <i>vs</i> sorafenib as first-line treatment in patients with advanced hepatocellular carcinoma	Nivolumab <i>vs</i> sorafenib	Recruiting

not result in longer OS or progression-free survival compared to sorafenib alone, although the primary endpoint (4-mo progression-free survival > 50%) was reached^[39]. Conventional cytotoxic chemotherapy has been investigated in a first line, phase III trial conducted in Asia and comparing the effects of oxaliplatin/fluorouracil with doxorubicin^[40]. Significant benefits of FOLFOX were found on progression-free survival, while OS resulted significant only in a post-hoc analysis (6.5 mo *vs* 4.9 mo). Sorafenib in combination with other chemotherapeutic regimens, *e.g.* gemcitabine/oxaliplatin or capecitabine/oxaliplatin is currently being investigated in phase II studies. Although the combination of cytotoxic chemotherapy and sorafenib is still being evaluated in clinical trials, this combination does not appear particularly promising.

Second-line

Patients who fail first-line systemic therapy are considered to have poor prognosis, and second-line trials are warranted^[41] (Table 3). Brivanib was also investigated in the BRISK-PS (brivanib-post sorafenib) trial, where brivanib and placebo were compared in patients who progressed on/after or were intolerant to sorafenib. Although TTP was significantly longer in the brivanib arm than with placebo (4.2 mo *vs* 2.7 mo), the primary end point of the study was not reached, as no differences in OS were observed comparing brivanib and placebo (9.4 and 8.2 mo, respectively)^[42]. It is possible that imbalances in patients' recruitment, favoring the placebo arm in terms of some parameters associated with a better prognosis, contributed to the failure of the BRISK-PS trial^[43].

The human anti-vascular endothelial growth factor Receptor 2 antibody, ramucirumab, has been recently studied in a second-line, phase III in comparison to

placebo^[44]. Median OS for the ramucirumab group was 9.2 mo *vs* 7.6 mo for the placebo group ($P = 0.14$), and thus the primary endpoint of the study was not reached. However, a subgroup analysis showed that patients with elevated alpha-fetoprotein could benefit from this treatment. Therefore, a phase 3, placebo-controlled trial testing ramucirumab as a second-line treatment in patients with elevated basal alpha-fetoprotein is currently recruiting patients (NCT02435433, clinicaltrials.gov, accessed April 25, 2016). Similarly, administration of everolimus to patients who failed sorafenib as a first-line treatment did not result in an improved OS over placebo (7.6 mo *vs* 7.3 mo)^[45]. Other mammalian target of rapamycin inhibitors have been tested in phase I - II trials, but conflicting results have been reported^[43].

Ongoing studies

Other compounds are currently under investigation in phase III trial, the final results of which have not been yet reported. These include other compounds acting as antiangiogenic agents, including lenvatinib, regorafenib and dovitinib. These are summarized in Table 4. For a more complete discussion of ongoing studies and additional targets refer to a recent comprehensive review^[43].

A promising approach has been obtained with the phase II study investigating tivantinib, an inhibitor of the Met tyrosine kinase, the receptor for hepatocyte growth factor. In this study, patients overexpressing Met, the target of tivantinib, had a significant benefit over placebo^[12]. Remarkably, expression of Met in patients receiving placebo was associated with a more aggressive behavior of the tumor, indicating that Met is both a therapeutic target and a prognostic biomarker. A phase III trial comparing tivantinib and placebo as a

second line therapy is currently underway. Along the same lines, a trial comparing placebo and cabozantinib, a dual Met and Hepatocyte growth factor inhibitor, has been undertaken.

One of the most promising areas in the field of HCC is represented by immunotherapy. Expression of PD-1 and CTLA-4 on immune cells is associated with blockade of the anti-tumor immune response, favoring the progression of cancer^[46]. In a Phase I/II study recently presented in abstract form nivolumab, an anti-PD-1 monoclonal antibody, induced tumor size stabilization or reduction in 67% of the patients^[47]. In addition, the effects of this treatment were durable, as previously observed in other types of cancer. A phase III study comparing the effects of sorafenib and nivolumab in advanced HCC is currently underway (Table 4).

Combination therapy

Sorafenib combined with classic chemotherapy: HCC is considered a poor responder to chemotherapy, which is not routinely used because of adverse events, particularly in patients with advanced cirrhosis. However, shrinkage of the tumor has been reported, although the magnitude of response is lacking consistency. This has led to the possibility to add sorafenib to a chemotherapeutic agent, as above reported, although the toxicity profile of any chemotherapeutic drug to be added to sorafenib should be kept in mind^[48,49].

Sorafenib and TACE could be a promising strategies in advanced HCC treatment. The high rate of HCC recurrence after TACE may be due to its enhancement of angiogenesis and upregulation of Vascular Endothelial Growth Factor and platelet-derived growth factor receptors expression, which increases tumor angiogenesis. Therefore, combination of antiangiogenic agents with TACE, could potentially decrease the recurrence of HCC and improve survival. A phase III study has been conducted in Japan and Korea using sorafenib in combination with TACE vs TACE alone. However, combination therapy failed to show any benefit in terms of TTP (sorafenib vs placebo 5.4 mo vs 3.7 mo) or OS^[50]. The results of the SPACE trial comparing sorafenib and placebo in patients undergoing TACE have been recently published. The combination of sorafenib plus TACE with drug-eluting beads was technically feasible, but the combination did not improve TTP in a clinically meaningful manner^[51].

ADVANCED HCC WITH PORTAL VEIN THROMBOSIS

Advanced HCC with portal vein thrombosis (PVT) has a very poor prognosis, and includes a special population of patients at higher risk of liver failure. Reported OS is about 10-24 mo in patients without PVT treated with BSC, compared to 2-4 mo in PVT patients^[52,53]. Clinical guidelines recommend sorafenib if PVT is present, but

different strategies such as surgery, TACE, external radiation therapy, Trans-Arterial-Radio-Embolization (TARE) and combination therapies are object of several clinical trials. The surgical option, frequently employed in Asia, does not show satisfactory results and it is often technically difficult and not safe (operative mortality rate until 6%) in these patients^[54]. TACE is also not recommended in PVT patients, because the injury due to ischemic events may cause serious complications like post-embolization syndrome and liver failure. Therefore TACE should be reserved to those patients with preserved liver function. The results in term of OS are good (from 7.4 mo to 10.2 mo) if compared with BSC, but in general they are not significantly better than those observed during sorafenib therapy^[55].

External radiation therapy is largely used in the treatment of cancer, but its role in HCC patients (with or without PVT) is very limited. Today, with newer techniques, a high dose of radiation can be delivered within the tumor, sparing normal liver parenchyma from radiation damage. Two large studies in China and Japan show that OS is longer in patients receiving radiotherapy compared to those treated with sorafenib or surgery^[56,57], but these data need to be confirmed in other settings.

Sorafenib is the only drug approved for HCC with PVT. Data from a sub-analysis of the two most important studies in United States and Asia show an OS of 8.1 mo vs 4.9 mo in control group) and 6.5 mo (vs 4.2 mo in control group), respectively. However Jeong *et al.*^[58], in a smaller study with 33 HCC patients with PVT, shows a percentage of stable disease and disease control rate lower than in the SHARP and Asia-Pacific studies. This data can be easily explained by the fact that in Jeong's study all patients had PVT (first order branches or main trunk), while in SHARP and Asia Pacific trials the percentage of macrovascular disease was much lower (about 36%). It is clear that the presence or absence of PVT negatively influence the prognosis. During treatment, no significant differences in OS have been shown between patients with thrombosis of first order branches and those with thrombosis of the main trunk^[58].

TARE is another important tool in the management of HCC with PVT. Some studies reported an OS ranging from 10 to 10.4 mo after treatment^[59,60]. Moreover, less adverse events have been reported and in general a better quality of life is shown if compared with TACE. TARE, the efficacy and safety of which has been widely tested in the last decade, is based on the administration of glass or resin microspheres loaded with a radioisotope (usually ⁹⁰Yttrium) through catheterization of the hepatic artery. The microspheres deliver a tumoricidal beta-radiation with a mean penetration of 2.5 mm, and a super-selective action towards tumoral tissue. As the embolic action is negligible, the procedure is generally well tolerated.

Table 5 Assessment of target lesion response: Conventional Response Evaluation Criteria in Solid Tumors and modified Response Evaluation Criteria in Solid Tumors assessment for hepatocellular carcinoma following the American Association for the Study of Liver Diseases-Journal of the National Cancer Institute guideline

RECIST	mRECIST
CR: Disappearance of all target lesions.	CR: Disappearance of any intratumoral arterial enhancement in all target lesions.
PR: At least a 30% decrease in the sum of diameters of target lesions, taking as reference the baseline sum of the diameters of target lesions.	PR: At least a 30% decrease in the sum of diameters of viable (enhancement in the arterial phase) target lesions, taking as reference the baseline sum of the diameters of target lesions.
SD: Any cases that do not qualify for either partial response or progressive disease.	SD: Any cases that do not qualify for either partial response or progressive disease.
PD: An increase of at least 20% in the sum of the diameters of target lesions, taking as reference the smallest sum of the diameters of target lesions recorded since treatment started.	PD: An increase of at least 20% in the sum of the diameters of viable (enhancing) target lesions, taking as reference the smallest sum of the diameters of viable (enhancing) target lesions recorded since treatment started.

mRECIST: Modified Response Evaluation Criteria in Solid Tumors; CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease.

TARE efficacy in HCC has been tested in several studies, although most of them were limited to a small number of patients. The largest observational studies reported a median OS in BCLC-C patients similar to that observed in the sorafenib arm of the SHARP trial^[59,61]. Additionally, a comparative study TACE vs TARE demonstrated the superiority of the latter in prolonging TTP but not in patients with advanced HCC^[62]. In a retrospective study, the efficacy of TARE has been compared to that of sorafenib, and no significant differences in OS were found^[63]. It should be considered that the study had several limitations, including a small sample size, and imbalance in baseline characteristics between the arms. On the contrary TARE appears to be particularly effective in patients with portal vein thrombosis, with a median OS ranging between 10-18 mo^[60,64,65] compared to 8.3 mo in patients with portal vein thrombosis treated with sorafenib in the SHARP study^[11]. Similar results showing the superiority of TARE vs sorafenib in patients with portal vein thrombosis have also been reported in another recently published study^[66]. Moreover, the efficacy of TARE (alone or in combination with sorafenib) vs sorafenib alone therapy is being evaluated in at least five ongoing clinical trials. The results will be available in the near future and will probably define the role of TARE in HCC therapy. By now, a good profile of tolerability was reported in the preliminary analysis of SORAMIC trial^[67].

IMAGING STUDIES IN PREDICTION AND EVALUATION OF RESPONSE TO THERAPY

In the management of patients with HCC, the role of imaging is crucial, not only to allocate a patient to a specific stage in the BCLC system, but also to check the efficacy of the treatment and to evaluate the progression of disease. Once a diagnosis is made, imaging with computed tomography (CT) or magnetic resonance (MR) scan is performed every 2-3 mo if the patient is receiving treatment with sorafenib^[68], the only approved agent for the treatment of advanced HCC^[11]. The lesions are usually evaluated applying the Modified Response Evaluation Criteria In Solid Tumors (mRECIST) parameters^[69], which have been proposed to improve and replace the previous system, known as RECIST 1.1. Lesion dimension was the only criteria considered by RECIST 1.1, which did not give any information about the viable portion of tumor, measured by the degree of contrast enhancement. While the RECIST 1.1 system is still adopted by some hepatologists, changes on tumor size alone is not considered appropriate to establish a prognosis and to correlate imaging with patients' survival. In fact it has been demonstrated that not all patients who have clinical benefit from antiangiogenic therapy have a dimensional reduction of target lesions.

On the other hand, not all progressive disease at imaging is linked to a shorter survival^[11,70]. Conversely, patients who will have benefit from therapy in term of survival, do not immediately show a reduction of tumor size, but more frequently the efficacy of therapy is correlated with some early intralesional decrease in cellularity and vascularization changes^[8,70,71]. Recently, a new evaluation criteria called Response Evaluation Criteria In Cancer of the Liver (RECICL) have been introduced^[72]. It is based on 2-directional measurement, by contrast enhancement CE-CT or dynamic MR, of tumors showing arterial enhancement, instead of only one measurement as requested in mRECIST. The great advantage these two last criteria, compared to conventional RECIST 1.1, is that both of them evaluate the contrast-enhancing portion of the tumor rather than the whole tumor. For this reason the presence of necrotic areas within the lesions is considered a sign of response. These concepts are summarized in Table 5. Moreover, RECICL criteria also consider the non-enhanced part of a target lesion: this may be useful to investigate hypovascular HCC. Even if progress has been done in order to establish patients response during antiangiogenic therapy, it is not clear how to manage those patients who have no benefit from treatment, because at now there is no approved second-line therapy. Moreover, the concept of "progressive disease" should be refined. In fact, according to mRECIST, there are many types

of “disease progression”, including lesion growth, presence of a new lesion, or a distant metastasis. At now, it is still under investigation if these different types of disease evolution have the same significance for patients in terms of prognosis.

The evaluation of the response is a subject of intense discussion, especially since molecularly targeted drugs like sorafenib have been introduced and routinely used in clinical practice. Dimensional parameters, largely used in imaging until now, are no longer appropriate now. In fact, it is clear that the goal for standard chemotherapeutic agent is to reduce tumor dimension, but this rule does not apply to antiangiogenic drugs. The new molecules predominantly act inhibiting angiogenesis, inducing tumor tissue necrosis and this may not have effect on the whole tumor dimension, and tumor size does not necessary decrease after therapy^[73]. For this reason, RECIST 1.1 criteria have been overcome by the introduction of mRECIST, based on the evaluation of the viable portion (enhanced part) of a target lesion. Several studies have compared the efficacy of RECIST 1.1, mRECIST and European Association for the Study of the Liver (EASL) criteria in evaluating response to loco-regional or systemic therapies in HCC patients^[74,75]. While mRECIST and EASL are considered reliable in assessing response to loco-regional therapies (for example TACE or TARE), there is no general agreement on their appropriateness in the evaluation of response during systemic therapy. In fact, loco-regional therapies often give predictable results, which consist in a well-defined and easy to measure area of necrosis. On the contrary, systemic therapies lead to the appearance of irregular and not homogeneous areas of necrosis, not easily defined and measurable^[76,77].

One additional promising method for the evaluation of response to therapy has been introduced by Choi. It was first applied to evaluation of therapy response of GISTs at PET assessment. Its use, based on contrast enhancement CT dimensional (measure of diameter of a lesion) and vascular (density expressed in HU on arterial phase CT) parameters, is currently under evaluation. In particular, according to these criteria, a reduction of 10% in tumor diameter or a reduction of 15% in intralesional density is considered as partial response to therapy^[78].

Recently volumetric studies have also been proposed as alternative to mRECIST and EASL, because the actual dimension of a tumor may not be exactly evaluated with simply a mono or bi-dimensional measure^[79]. According to recently published studies focused on HCC patients, a 10% increase in volume rate after two months of therapy correlates with a poor prognosis^[80,81]. The role of MR diffusion weighted imaging has also been investigated in response assessment. Early variation (at first decrease, and subsequently increase) in apparent diffusion coefficient values after therapy seems to correlate with a better response. Moreover, low pre-treatment apparent diffusion coefficient values seem to

be predictive of a good response^[82,83]. On the contrary, the results of MR diffusion weighted imaging in HCC patients during therapy (loco-regional, systemic or combination therapies) are controversial and not yet clear because of low reproducibility of this technique.

Perfusion-weighted imaging is a relatively new MR/CT technique for qualitative and quantitative evaluation of the delivery of blood to biological tissues. Recently, attention has been focused on the “mean intralesional transit time”, which is the time that a contrast agent takes to go through the tissue volume (e.g. liver) from entry to exit^[84]. In several studies, this parameter showed not only good correlation with response to therapy, but its baseline value (before starting therapy) seems to be predictive of response. In fact some authors have shown that partial or complete responders had higher mean intralesional transit time levels at baseline examination compared to those with progressive disease^[85]. Both diffusion and perfusion techniques are still to be considered “research methods” not applicable in the clinical setting.

When evaluating the response to TARE, it should be considered that radiologic findings are more heterogeneous and variable than in other loco-regional treatments, and identification of residual disease, reactionary changes or complications is crucial to assess tumor response. After TARE the responding tumor can show shrinkage (diameters reduction), “vanishing” (enhancement decrement after contrast agent administration), and necrosis. In addition, various collateral findings can be observed, which could make even more difficult the response evaluation. These include perivascular edema, ring enhancement in case of coagulative necrosis, hepatocyte depletion and hepatic fibrosis^[86].

RECIST criteria are not always appropriate in evaluating response after TARE, and criteria which evaluate viable portion of a lesion like EASL and mRECIST are more suitable. In fact, according to Keepke and Seyal, both mRECIST and EASL showed superiority in evaluating objective response to TARE when compared with RECIST^[87,88]. On the other hand, other authors compared RECIST, mRECIST, Choi and mChoi criteria, showing that Choi and mRECIST are the most appropriate in assessing response after TARE. In particular, patients who have response according to Choi criteria have significantly longer TTP and OS, while non-responders have worse prognosis^[89]. Even if Choi criteria have shown good correlation between imaging and patient outcome, evaluation of tumor density applying a ROI within a lesion is not unanimously accepted, for the excessive inter and intra-observer variability^[90]. Moreover, measurements of density could be difficult in hypodense lesions and make this method not reliable.

Volumetric studies also can help the clinician to evaluate response to TARE. This technique is used in order to measure both the whole tumor and the necrotic area. According to Monsky *et al.*^[91] volumetric

technique is more suitable to evaluate dimension of a necrotic area after TARE. In addition, patients whose change in necrotic area is > 10% has longer survival if compared to patients whose change is < 10%.

PROGNOSIS IN ADVANCED HCC

As described before, advanced HCC is a condition where multiple actors can play a determinant role, resulting in large variability of the disease even in the same BCLC stage.

Portal vein thrombosis

The presence/absence of portal vein thrombosis and its extension, as well as extra-hepatic spread and alterations in liver function, can jeopardize the efficacy of specific treatments. Moreover, the natural history of the disease - even in absence of treatment - is strictly related to these variables. Finally, both natural history and the response to treatment may be influenced by molecular characteristics of the tumor. It is easy to understand how talking of prognosis "in general" for advanced HCC - as well as for all stages of HCC - sounds simplistic.

The natural history of the disease is difficult to evaluate through randomized controlled trials for ethical reasons. Nonetheless, some interesting studies have tried to clarify the prognosis of untreated HCC. A meta-analysis published in 2010 evaluated more than 4000 patients included in the placebo or inactive treatment arms of 30 randomized control trials in order to estimate survival in untreated HCC patients and to evaluate factors related to a different survival^[92]. The 1-year survival rate in BCLC B + C patients was 34%, with a pooled estimate 1-year survival of 25% in the subgroup of advanced HCC patients. ECOG performance status, albumin levels, prothrombin activity, portal vein thrombosis and Child Pugh score A emerged as predictors of longer survival in all HCC untreated patients. In the BCLC B + C group ECOG performance status, presence of ascites and an Okuda stage I were significantly related with a longer survival. A more recent retrospective cohort study evaluated 320 untreated HCC patients, 39% in advanced stage according to BCLC^[93]. The 1-year survival rate for advanced HCC patients was 12%, with a median survival of 6.9 mo. ECOG performance status, INR and alpha-fetoprotein emerged as independent predictors of mortality at multivariate analysis.

Distant metastases

A related emerging issue, analyzed in recent studies, has been the attempt to establish a correlation between progression and survival in patients with HCC. In order to do that, attention has been focused not only on classic OS but also on two new parameters, which were not considered in the past studies on HCC during systemic therapy. The first is TTP, defined as

the time from the date of starting therapy to disease progression, evaluated by imaging (CT or MRI). The second is the post-progression survival, which is the time from disease progression to death. Along these lines, four different kinds of progression (progression patterns) have been established: intrahepatic or extrahepatic tumor growth (> 20% increase in tumor size of viable target lesion), new intrahepatic lesion and new extrahepatic lesion (including new metastasis and/or vascular invasion). According to data reported by Lee *et al.*^[28], patients with only metastatic disease have a better post-progression survival than those with vascular invasion or both of them (respectively 7.7, 3.8 and 3 mo), probably because of a higher rate of liver failure in patient with vascular invasion. TTP also seems to be related with survival: in fact patients with early radiologic progression during sorafenib treatment have a much shorter survival than progressive disease patients at 4 mo (respectively 4.9 and 16.6 mo)^[28].

Similar results in term of survival had already been reported by Reig *et al.*^[94], who showed how the progression pattern may impact on prognosis. In particular the presence of new extrahepatic lesions and/or vascular invasion appear to be correlated to a shorter post progression survival. The purpose of correlating pattern progression with survival is to identify those patients who are eligible for second line treatment, and to appropriately stratify them. In order to do that, the concept of "BCLC upon progression", which evaluates the progression pattern of PD patients, has been introduced. In advanced HCC patients (BCLC-C) two kinds of progressions have been identified: patients who show an increase in size of an existing lesion or a new intrahepatic lesion (probably candidates for second line treatment) and patients who show extrahepatic lesions or vascular invasion, associated with a poor prognosis^[94].

BEST SUPPORTIVE CARE

There are currently very few data about BSC in advanced HCC. Although this issue has been neglected, it represents a very important aspect of the care of these patients, as much as in other patients with advanced cancer. According to EORTC "supportive care for cancer patients is the multi-professional attention to the individual's overall physical, psychological, spiritual and cultural needs, and should be available at all stages of the illness, for patients of all ages, and regardless of the current intention of any anti-cancer treatment"^[95]. According to BCLC, BSC is the only treatment option in terminal stage HCC. However, the definition of BSC implies its application during every stage of the disease. Despite its importance, BSC is marginally discussed or even only mentioned in all guidelines. The goal of BSC is to improve the quality of life, which is obviously reduced in patient with HCC compared to the general population^[96]. BSC should

be performed in order to avoid the complications of cirrhosis (ascites, gastro-intestinal hemorrhage, encephalopathy, hepatorenal syndrome, spontaneous bacterial peritonitis), but should also be focused to those conditions which are typical of oncologic patients^[97]. The most common symptoms reported by HCC patients are sleep disturbances, depression, fatigue, malnutrition, anorexia, pain and psychological issues^[98-101].

Unsatisfactory night sleep is reported by 50%-65% of patients with cirrhosis, that actually reduces sleep time, sleep efficacy and REM sleep and increase sleep latency. These pathological changes correlate with the grade of liver dysfunction^[102-105]. Insomnia is also reported by 50%-65% of cirrhotic patients and excessive daytime sleepiness is part of the hepatic encephalopathy syndrome^[105,106]. Physicians should perform a routine assessment of sleep quality and time and evaluate daytime sleepiness. If the latter is present a treatment for hyperammonemia should be started or increased. Moreover, sleep and light hygiene practices (regular sleep-wake schedule, exposure to bright light in the morning and not in the evening) should be encouraged. While hypnotics should be used with caution, 25 mg hydroxyzine before sleeping has induced a subjective sleep improvement compared to placebo with a good profile of tolerability^[107].

Depression and anxiety are major determinants of an altered quality of life, even after a curative treatment, and are reported in more than 60% of HCC patients. Treatment should include supportive psychotherapy or behavioral interventions, with particular attention for the relationship between physician and patient and his family. Liver dysfunction modifies pharmacokinetics of antidepressants, which should be introduced at low dosage (1st line citalopram, sertraline)^[108,109]. If no improvement is observed in 2 wk, dosage should be increased. A switch to a second line therapy (e.g., paroxetine) should be performed after 4-6 wk of therapy without improvement of symptoms. All psycho-social and spiritual issues are particularly frequent in end-stage disease and require a careful approach, both pharmacological and supportive. Fatigue is very frequent in HCC patients and may be related to multiple causes: depression, sleep disturbances, cachexia, anemia. Exercise, whose level must be related to fatigue, significantly reduces cancer-related fatigue during and after the treatment^[110]. Although no specific data are available for HCC, administration of modafinil (a non-amphetamine-based stimulant) has recently shown a significant improvement in fatigue in a trial vs placebo involving 631 cancer patients^[111].

Malnutrition, anorexia and cachexia are related to the tumor and to the weight loss and muscle wasting observed in cirrhosis. Sarcopenia, that is frequent in alcoholic and cholestatic diseases and may be related to portosystemic shunting, also contributes to malnutrition and cachexia. An adequate energy uptake, exercise

and the avoiding of unnecessary diet restriction such as a low protein diet should be recommended. Few and controversial data on parenteral support are available^[112]. A randomized controlled trials by Chow *et al*^[113] showed that megestrol acetate improved emotional functioning, nausea, vomiting and appetite loss in patients with HCC, while no benefit was observed in OS or quality of life^[114]. Numerous studies evaluated the role of oral branched-chain amino acid administration in HCC patients, although very few data are available in the subset of advanced HCC. In general, this kind of treatment seems to improve liver function, and malnutrition (with a significant increase of albumin levels), but no clear effect has been observed on OS^[114-117], even though a recent meta-analysis showed an improvement in the 3-years mortality^[118]. Although more data are needed to confirm its efficacy, oral branched-chain amino acid supplementation may be considered in HCC patients to improve the liver reserve and quality of life.

Treatment of pain in HCC varies according to the cause. Bone metastases-related pain can be treated with cementoplasty^[119] or irradiation. Irradiation can also be used for the treatment of painful lymph nodes and lung metastases. In a recent phase II trial by Soliman *et al*^[120] liver radiotherapy showed promising results in symptom improvement at one month. In symptomatic treatment nonsteroidal anti-inflammatory drugs should not be used, due to the possibility of hepatorenal syndrome, hepatotoxicity, and gastro-intestinal bleeding. Acetaminophen 2-3 g daily is the first line agent in long-term use, while opioids should be used as second line treatment. Liver participates in degradation and biotransformation to active metabolites of opioids, so a good knowledge of their pharmacokinetics is mandatory. Hydromorphone and fentanyl should be preferred, as they are least affected by renal dysfunction. Treatment should be started with low dose and a 2-3 d titration, with a regular assessment of efficacy and tolerance. Long acting agents should be preferred, possibly in association with a short active drug and paracetamol and/or corticosteroids. A dose increase of 20%-30% must be performed when necessary^[121].

Muscle cramps are very frequent in patients with cirrhosis and HCC and may be related to electrolyte imbalance, that must be treated. Many agents showed positive results in muscle cramps treatment in cirrhotic patients, but there is still need of controlled trials. The most interesting agents are taurine, whose synthesis is reduced in cirrhosis leading to a decrease in membrane stabilization, and quinine sulfate^[122]. Baclofen is also used by some physicians due to its skeletal-muscle relaxant activity. The drug was reported as effective and safe in a pilot trial including 10 cirrhotic patients^[123] and is currently being tested in a randomized controlled trials (Baclofen in the Treatment of Muscle Cramps in Patients With Cirrhosis, ClinicalTrials.gov Identifier: NCT02221570).

CONCLUSIONS AND PERSPECTIVES

The ability to treat earlier stages of HCC and the longer survival of patients with cirrhosis make advanced HCC a common problem facing the Hepatologist. In the past few years a breakthrough step has been the approval of sorafenib as a systemic therapy for this type of cancer. However, we still lack reliable early predictors of the likelihood to respond to sorafenib, to be utilized at the single patient level. Unfortunately, sorafenib has not been followed by approval of other drugs for use in first- or second-line treatment of advanced HCC. Moreover, the role of other approaches to the treatment of the advanced stage, including TARE, conformational radiotherapy, and conventional chemotherapy deserve additional investigation. Attention is being focused on the significance of different types of advanced HCC, *e.g.*, due to the presence of extrahepatic spread or to involvement of the portal vein. Along these lines, the type of progressive disease which leads to migration to an advanced stage plays a yet unknown but probably important role. These lines of information need to be integrated with accumulating data on the molecular heterogeneity of HCC. Collectively, these data will be instrumental to design personalized treatments, considering that HCC is one of the few solid tumors where no molecular-guided therapy exists. Finally, more attention to supportive care needs to be paid by Hepatologists dealing with patients in advanced or terminal stages of the disease, including initiation of supportive treatment and avoiding delay in withdrawing active therapies when unnecessary. Thus, active research in this field will hopefully lead to an even better management of these difficult-to-treat patients.

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Exploring the metabolic syndrome: Nonalcoholic fatty pancreas disease

Roberto Catanzaro, Biagio Cuffari, Angelo Italia, Francesco Marotta

Roberto Catanzaro, Biagio Cuffari, Angelo Italia, Department of Clinical and Experimental Medicine, Gastroenterology and Hepatology Service, Internal Medicine Unit, University Hospital Policlinico "G. Rodolico", University of Catania, 95123 Catania, Italy

Francesco Marotta, ReGenera Research Group for Aging-Intervention, 20144 Milano, Italy

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Correspondence to: Roberto Catanzaro, MD, PhD, Department of Clinical and Experimental Medicine, Gastroenterology and Hepatology Service, Internal Medicine Unit, University of Catania, Policlinico "G. Rodolico", Via S. Sofia 78, 95123 Catania, Italy. rcatanza@unict.it
Telephone: +39-95-3782902
Fax: +39-95-3782376

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Abstract

After the first description of fatty pancreas in 1933, the effects of pancreatic steatosis have been poorly investigated, compared with that of the liver. However, the interest of research is increasing. Fat accumulation, associated with obesity and the metabolic syndrome (MetS), has been defined as "fatty infiltration" or "nonalcoholic fatty pancreas disease" (NAFPD). The term "fatty replacement" describes a distinct phenomenon characterized by death of acinar cells and replacement by adipose tissue. Risk factors for developing NAFPD include obesity, increasing age, male sex, hypertension, dyslipidemia, alcohol and hyperferritinemia. Increasing evidence support the role of pancreatic fat in the development of type 2 diabetes mellitus, MetS, atherosclerosis, severe acute pancreatitis and even pancreatic cancer. Evidence exists that fatty pancreas could be used as the initial indicator of "ectopic fat deposition", which is a key element of nonalcoholic fatty liver disease and/or MetS. Moreover, in patients with fatty pancreas, pancreaticoduodenectomy is associated with an increased risk of intraoperative blood loss and post-operative pancreatic fistula.

Key words: Metabolic syndrome; Nonalcoholic fatty liver disease; Pancreatic steatosis; Pancreatic lipomatosis; Nonalcoholic fatty pancreas disease; Fatty pancreas; Pancreatic fat; Pancreatic fatty replacement; Pancreatic fatty infiltration; Pancreatic cancer

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Core tip: Nonalcoholic fatty pancreas disease is a very common yet neglected pathological condition. It can be considered an early marker of the metabolic syndrome and, as so, its clinical significance spaces between internal and surgical diseases, such as type 2 diabetes mellitus, atherosclerosis, acute pancreatitis and even pancreatic cancer. This review collects current knowledge of worldwide opinion leaders and

researchers of this matter.

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INTRODUCTION

The spreading of obesity is one of the most concerning problems of modern medicine. According to the World Health Organization (WHO), worldwide obesity has nearly doubled since 1980, and in 2008 more than 10% of the world's adult population was obese^[1]. About 3.4 million adults die every year because of overweight or obesity, which are more deaths than underweight.

According to the International Diabetes Federation, the association of abdominal (central) obesity with hypertension, elevated fasting plasma glucose, high serum triglycerides, and low high density lipoproteins (HDL) define the metabolic syndrome (MetS), also known as syndrome X, cardiometabolic syndrome or insulin resistance syndrome. This condition is associated with a pro-inflammatory, pro-thrombotic state, and leads to an increased risk of developing cardiovascular disease and type 2 diabetes mellitus (T2DM)^[2,3].

In the last years, more research is focusing on understanding obesity, MetS and the diseases associated.

Obesity, especially when associated with a higher waist circumference, causes ectopic fat deposition in certain organs, such as the liver (nonalcoholic fatty liver disease - NAFLD), heart, muscles, kidney and pancreas^[4]. This is called steatosis^[5].

As the liver is a key organ in the metabolism, its fatty infiltration has been the most investigated. Large evidence supports the hypothesis of NAFLD as both cause and consequence of the MetS^[6].

Although the pancreas is also an important organ in the metabolism, the effects of fatty infiltration of this organ has been less investigated than that of the liver.

RESEARCH

The following research was performed on MEDLINE/PubMed: "pancreatic steatosis" or "pancreatic lipomatosis" or "NAFPD" or "fatty pancreas" or "pancreatic fat" or "pancreatic fatty replacement" or "pancreatic fatty infiltration". A total of 210 results were found and abstracts were examined. Thirty-four papers were excluded because the main topic was not pancreatic fat. While reviewing, further references were added.

HISTORY

The first description of pancreatic fat was made by

Table 1 Nomenclature according to Smits and van Geenen^[9]

Name	Definition
Pancreatic steatosis	General term for pancreatic fat accumulation
Pancreatic lipomatosis	
Fatty pancreas	
Lipomatous pseudohypertrophy	
Fatty replacement	Extreme variant of pancreatic fat accumulation when the pancreas is enlarged uniformly or focally, the exocrine system is replaced by fat, and when no association can be found with obesity ^[10]
Fatty infiltration	Death of acinar cells with subsequent replacement with adipocytes
NAFPD	Infiltration of adipocytes owing to obesity
NASP	Pancreatic fat accumulation in association with obesity and metabolic syndrome
	Pancreatitis owing to pancreatic fat accumulation

NAFPD: Non-alcoholic fatty pancreas disease; NASP: Non-alcoholic fatty steatopancreatitis.

Ogilvie in 1933^[7]. He compared 19 pancreas derived from obese patients with 19 controls. Obese cadavers showed a greater mean pancreatic adiposity (17.1%, range 0%-48.5%) than the controls (9.3% range 2.5%-23.6%).

After more than 40 years, in 1978, Olsen^[8] performed a larger study over 394 autopsies. The cadavers were divided into three groups: below normal weight, normal weight and above normal weight. He found a relationship between the content of fat and age, and confirmed the relation with obesity.

Across the years, many synonymous have been used to refer to "pancreatic fat accumulation", with many different meanings. Those terms have been very well reviewed in 2011, and are summarized in Table 1. According to the authors, the limit of this nomenclature is the lack of distinction between the accumulation of triglycerides in acinar cells, β -cells or intrapancreatic adipocyte tissue^[9,10].

Nowadays, with the development of more sophisticated imaging techniques and data suggesting the clinical importance of obesity and the MetS, the interest of researchers is increasing, as shown in Figure 1.

HOW TO ASSESS PANCREATIC FAT

Anatomical pathology

Mild or massive pancreatic steatosis can be assessed by simple inspection of the organ. That can be useful in the surgical setting, as further explained^[11].

Histological examination is the most common way to assess pancreatic fatty infiltration in animal models^[12-28]. Human specimen can be obtained from autopsies, operatorily remnants or, rarely, fine needle aspiration cytological (FNAC) examination^[7,8,16,29-40].

Fat accumulation may be even or uneven^[41-43]. Four different types of uneven pancreatic lipomatosis have been described: (1) Type 1a (35% of cases):

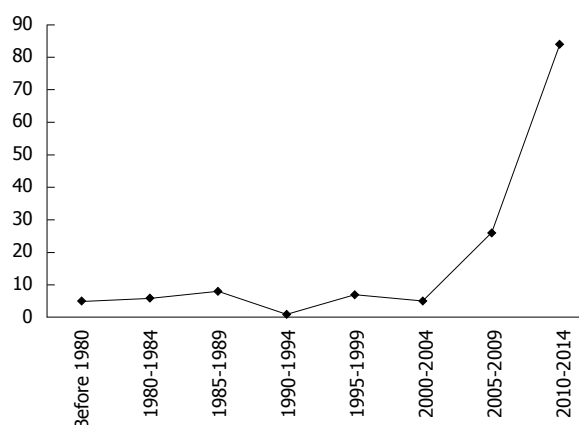


Figure 1 MEDLINE/PubMed findings about pancreatic fat.

replacement of the head with sparing of the uncinate process and peribiliary region; (2) Type 1b (35%): replacement of the head, neck, and body, with sparing of the uncinate process and peribiliary region; (3) Type 2a (12%): replacement of the head, including the uncinate process, and sparing of the peribiliary region; and (4) Type 2b (18%): total replacement of the pancreas with sparing of the peribiliary region^[41,44].

Unlike the liver, where the triglycerides accumulation is mainly intracellular, pancreatic steatosis is histologically characterized by an increased number of adipocytes (Figures 2 and 3)^[5,16]. However, intracellular fat accumulation can be visualized by electronic microscopy or immunohistochemistry in both acinar and islet cells and may precede adipocytes infiltration^[12,13,16,18,21,23,45-48]. It is unknown if intracellular or extracellular triglycerides have a different clinical significance, but it is possible that adipocytes influence the function of acinar and/or islet cells by a paracrine effect, while intracellular lipids may lead to lipotoxicity and therefore islet or acinar cells injury, as further discussed^[9].

Thus now, there is not a shared score to grade the severity of fatty infiltration on histological examination, so each group have used arbitrary subjective parameters, or computer-based morphometric analysis, which gives an objective quantification of pancreatic fat^[8,30,31,33,34,38,48-50].

To our knowledge, the only scoring system that has been validated on patients by a rastering method is the pancreatic lipomatosis score (PLS), developed by van Geenen *et al.*^[9,33].

PLS modifies the classification made by Olsen in 1978^[8], based on the percentage of adipocytes per microscopic field: (1) Group 1: $\geq 51\%$; (2) Group 2: $\geq 26\%$; (3) Group 3: $\geq 15\%$; and (4) Group 4: $\geq 8\%$.

The group numbers were shifted and a group for patients with less than 8% fat was added. Furthermore, intralobular fat, interlobular fat and total fat were scored separately. A last group was added for pancreases with $> 75\%$ of total fat.

Radiological assessment

The majority of radiological techniques available have been used to study pancreatic steatosis. So far, there is not cut-off points validated on patients, nor valid comparative trials that are able to assess which technique is the most accurate.

Ultrasonography: Both transabdominal ultrasound and endoscopic ultrasound (EUS) can be used to observe the pancreas^[42,51-66]. A fatty pancreas looks hyperechogenic (hyperechogenic pancreas - HP) compared to the liver or, if the liver is also hyperechogenic, with the spleen or the kidney. Since the kidney and the pancreas cannot often be seen in the same window, one could use an indirect comparison between the kidney with the liver, and then the liver with the pancreas^[57,58].

By EUS, the echogenicity of the pancreas can also be compared with the one of retroperitoneal fat^[65]. In addition, EUS may also be associated with FNAC for cytological analysis^[36].

Some authors have even graded the severity of pancreatic fat infiltration basing on echogenicity only, or on a grading system based on the aspect of the pancreatic duct and the presence of parenchymal "salt and pepper" dots^[56,59].

Transabdominal ultrasound is cheap, fast and non-invasive, but the pancreas can't always be visualized, especially in obese patients. Another limitation of both transabdominal and endoscopic ultrasonography is that they are operator dependent.

More important, HP may not be a certain indicator of pancreatic fat infiltration, as a fibrotic pancreas is also hyperechogenic^[60]. Therefore, it has been suggested that ultrasonography (US) shouldn't be used as a screening tool for pancreatic fat content, and that computed tomography (CT) or magnetic resonance imaging (MRI) should be the second step to confirm the diagnosis^[9,60].

CT: CT imaging is widely used to study all abdominal organs. A fatty pancreas will be hypodense in hounsfield units (HU) compared to the spleen^[39,67-69]. In severe fatty replacement, the attenuation can be compared with the adjacent retroperitoneal fat^[42]. When the condition is severe, differentiation between lipomatosis and pancreatic agenesis can be made by evaluating the ductal system, which will be present in fatty replacement and absent in agenesis^[44]. Unenhanced CT should be preferred, as the normal pancreatic parenchyma between fatty areas could exhibit contrast enhancement, and focal fatty replacement could simulate a true mass^[70].

To validate CT for the diagnosis of pancreatic steatosis, Saisho *et al.*^[30] showed that the fat/parenchyma ratio calculated from CT is analogue to histological evaluation. Moreover, in 2014, Kim *et al.*^[39] found

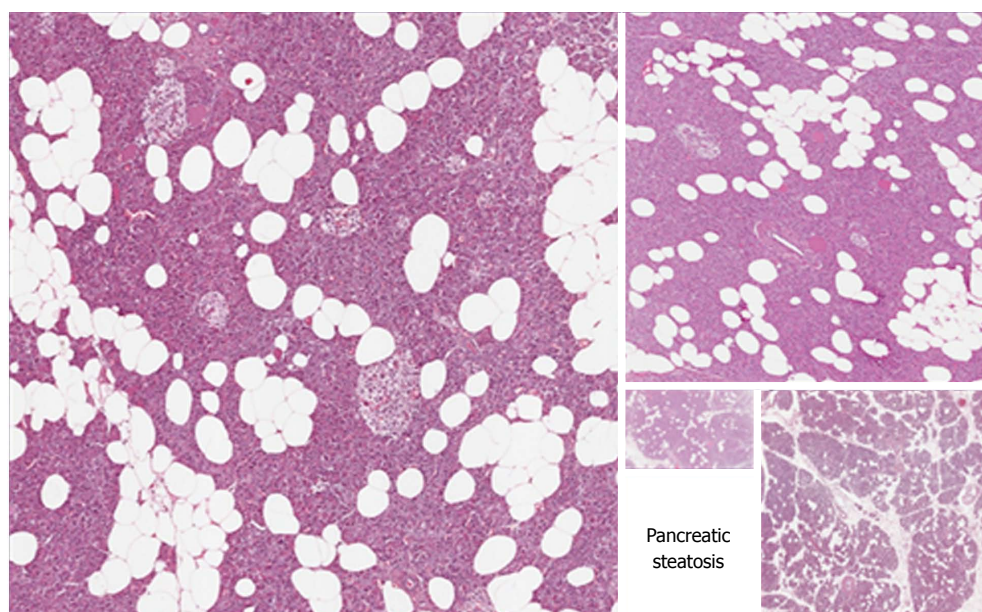


Figure 2 Pancreatic steatosis. Courtesy of Prof. Vasquez E and Dr. Angelico G, Anatomical Pathology Department, University of Catania -Catania, Italy.

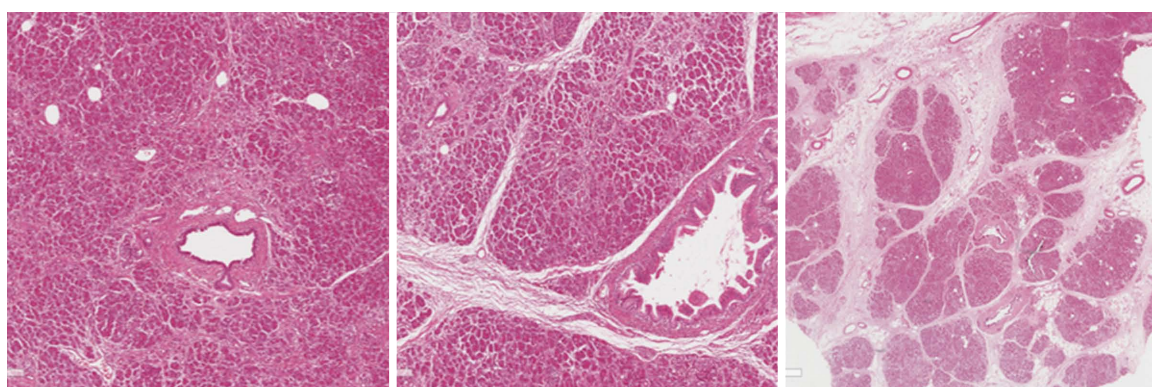


Figure 3 Normal pancreas. Courtesy of Prof. Vasquez E and Dr. Angelico G, Anatomical Pathology Department, University of Catania -Catania, Italy.

that pancreatic fat fraction in histological evaluation was significantly correlated with the difference between pancreatic and splenic attenuation ($P < 0.01$) and the pancreas-to-spleen attenuation ratio ($P < 0.01$).

The use of ionizing radiation limits CT as a research method, but recent evidence suggests that preoperative CT evaluation of pancreatic fat may be of importance in predicting the clinical outcome in pancreatic surgery, or as a prognostic marker for pancreatic adenocarcinoma^[67,68,71].

MRI: MRI is sensible, noninvasive and safe. For those reasons, it's currently the most used method to study fat content of the pancreas, especially in prospective studies. Single-voxel magnetic resonance spectroscopy (MRS) is considered almost equivalent to histology and biochemical measurements, and therefore is currently the criterion standard for the determination of pancreatic lipomatosis^[72-79].

There are several methods to measure pancreatic fat fraction (PFF) in the pancreas using MRI.

The most used method utilize the frequency shift between the water and the fat resonances to generate in-phase and opposed-phase images, in which the signal of the water and fat net magnetization vectors are at a maximum or a minimum^[80].

The Dixon method consist of a post-processing of the in-phase and opposed-phase spin echo images that uses the chemical shift difference between protons in water and fat, leading to water-selective and fat-selective images^[81]. However, the results can be affected by T1 and T2 relaxation effects^[82]. The novel and fast two-point mDixon exhibits a good correlation with MRS for assessment of PFF, with limited sensitivity for assessing lower fat content^[76].

Spectral-spatial excitation technique combine chemical shift selectivity with simultaneous slice-selective excitation in gradient-echo imaging sequences. Apparently, this method is as good as in-phase/opposed-phase imaging on determinate the PFF^[80], and is particularly good for determining small amounts of fat^[83].

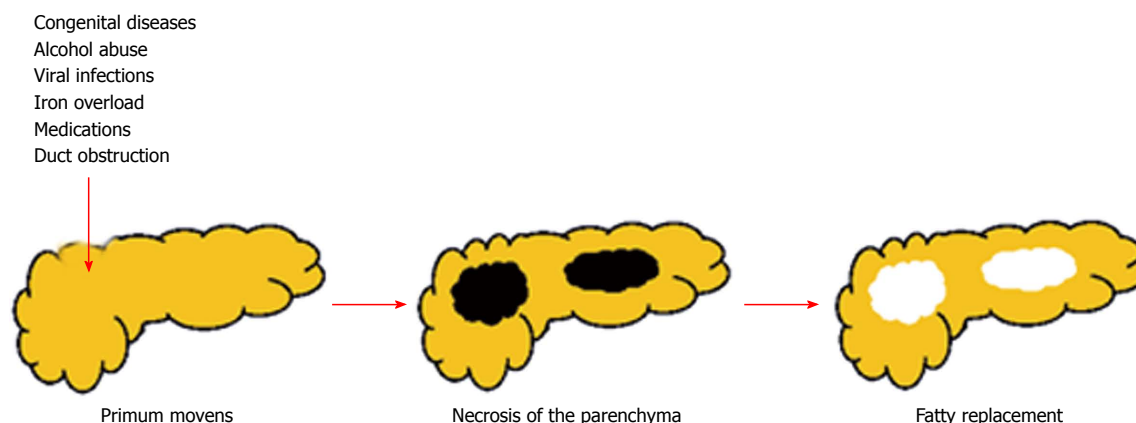


Figure 4 Pathogenesis of "fatty replacement".

A recently developed method is the three dimensional iterative decomposition with echo asymmetry and least squares estimation (IDEAL), which produces separated fat and water images, optimal in signal-noise ratio. Hu *et al*^[73] reported that this method may be even superior to MRS in the measurement of PFF.

Finally, the newest automated intra-subject registration-based segmentation is potentially suitable for the quantification of abdominal and organ fat and achieves comparable quantitative endpoints with respect to manual segmentation^[84].

EPIDEMIOLOGY

Only few epidemiologic studies have been performed to assess the prevalence of pancreatic steatosis. The estimated prevalence is between 16% and 35% in Asian populations^[64,85,86].

In 2016, Pham *et al*^[87] published the first study on which the prevalence of pancreatic steatosis was assessed in a pediatric population, which was 10%. However, this result may not be extended in general pediatric population, as it was assessed on hospitalized patients.

However, even considering the limits of those studies, all of them suggest a large prevalence in general population.

PATHOGENESIS AND RISK FACTORS

There are at least two mechanisms that can lead to a pancreatic fat accumulation^[9]: (1) death of acinar cells and replacement by adipocytes - "fatty replacement" (Figure 4); and (2) fat accumulation associated with obesity and type 2 diabetes mellitus - "fatty infiltration" or "NAFPD".

Fatty replacement

Theoretically, any noxa strong enough to cause necrosis of the acinar cells can lead to fatty replacement^[9]. Despite that, only little evidence can be found in literature.

Congenital diseases: Cystic fibrosis (CF) or mucoviscidosis is an autosomal recessive disorder. It is caused by the presence of mutations in both copies of the gene for the protein cystic fibrosis transmembrane conductance regulator (CFTR), involved in the production of pancreatic juice. CF results in a more dense pancreatic secretion, which eventually leads to pancreatic damage and replacement with adipocytes^[88-101].

Shwachman-Diamond syndrome or Shwachman-Bodian-Diamond syndrome (SBDS) is a rare autosomal recessive disorder characterized by exocrine pancreatic insufficiency with fatty replacement, bone marrow dysfunction, skeletal abnormalities, and short stature. The gene mutated in this syndrome is called SBDS, and its function is probably involved in RNA metabolism or ribosome assembly, although it's uncertain. Therefore, the pathogenesis of pancreatic damage is still unclear^[51,102-108].

Johanson-Blizzard syndrome (JBS) is caused by mutations in the *UBR1* gene, which encodes one of several ubiquitin ligase enzymes of the N-end rule pathway. It is associated with developmental errors, impaired apoptosis, and both prenatal and chronic inflammatory damage, necrosis and fibrosis of the pancreatic acini. Pancreatic exocrine insufficiency in JBS can additionally stem from congenital replacement of the acini with fatty tissue^[109-114].

Finally, heterozygous carboxyl-ester-lipase mutations are associated with fatty replacement of the pancreas and maturity onset diabetes of the young, probably due to a protein misfolding^[55,115].

Those genetic conditions can also be associated with exocrine pancreatic insufficiency (EPI), as a result of the destruction of exocrine pancreatic parenchyma.

Alcohol abuse: It has been suggested that alcohol abuse may be associated with abnormal mitochondrial function, which may account for the fat accumulation observed in pancreatic acinar cells^[116]. However, evidence don't support this theory, and it is more probable that alcohol abuse leads to pancreatic lipomatosis *via* acute and/or chronic pancreatitis and/or

the upregulation of transcription factors involved in the synthesis of cholesterol and triglycerides^[12,13,57,59,116-118]. Also, alcoholism is associated with malnutrition, which is also a cause of pancreatic steatosis^[13].

Viral infections: Viral infections with Reovirus, may lead to duct obstruction and therefore necrosis of the parenchyma and subsequent substitution with fatty tissue^[9,49,119]. In support of this pathogenic pathway, pancreatic duct ligation actually leads to fatty replacement^[120,121].

Iron overload: The most important causes of iron overload are hereditary hemochromatosis, a genetic disorder, and transfusional iron overload, which can result from repeated blood transfusions. Iron mainly accumulates in reticuloendothelial system, liver, heart, and endocrine or exocrine glands, pancreas included. When the pancreas is involved, iron leads to oxidative stress of acinar and islet cells, apoptosis and substitution with adipocytes. This has been described in patients with transfusion-dependent diseases as myelodysplastic syndrome and cooley's anemia (β -thalassemia major)^[122-125].

Medications: Although it's theoretically valid, evidence that medications can cause pancreatic tissue necrosis and subsequent substitution with fatty tissue is scarce and mainly based on case reports or animal models. drugs reported are corticosteroids, gemcitabine, rosiglitazone and, more recently, octreotide^[17,49,126-130].

Chronic liver disease: Yoshimura *et al.*^[131] and Sasaki *et al.*^[132] suggested that lipomatous pseudohypertrophy of the pancreas might be caused by chronic advanced hepatic lesions, which lead to ductal obstruction. Thus now, only case reports of patients with chronic hepatitis B and liver cirrhosis support this hypothesis^[49,132,133].

Stronger evidence exists that pancreatic steatosis is associated with NAFLD^[33,56,57,59,64,75,134-137]. However, the pathogenesis of this association is more likely due to metabolic pathways than pancreatic injury, and it will be discussed in the appropriate section.

Malnutrition: Malnutrition, as seen in alcoholism, kwashiorkor and AIDS is associated with changing in pancreatic structure, including pancreatic lipomatosis^[13,29,138]. However, the lack of evidence makes the pathogenesis still unclear.

Pancreatitis: It is theoretically possible that necrotizing pancreatitis leads to fatty replacement, but, to our knowledge, this association has never been reported. In contrast, Recurrent Acute Pancreatitis (RAP) may lead to reduction of the parenchymal mass and substitution with adipocytes^[139-141]. Moreover, an increased number of intrapancreatic adipocytes can be observed in pancreata of lean patients with

nonhereditary or hereditary chronic pancreatitis^[142-144].

Moreover, NAFLD has been associated with an increased risk of developing severe acute pancreatitis, as discussed in the appropriate section.

Nonalcoholic fatty pancreas disease

The most important risk factor for developing NAFLD is obesity. This relation was suggested in the first study by Ogilvie and has been widely confirmed^[145]. Experimental models report that maternal obesity and postnatal obesogenic diets can result in a NAFLD by inducing an endoplasmic reticulum imbalance and alteration in circadian metabolic patterns^[25,146]. In addition to obesity, studies on sufficiently wide populations (> 1000 subjects) suggest also increasing age, male sex, hypertension, dyslipidemia, alcohol consumption, low serum lipase activity as important risk factors, although data must be considered still insufficient^[30,64,86,147]. T2DM and NAFLD are often reported as risk factor. However, data are sometimes contrasting, as further discussed. Wong *et al.*^[148] found also a positive correlation between fatty pancreas and hyperferritinemia.

Recently, different ethnicity has been suggested as an independent risk factor of developing pancreatic steatosis. Hispanics and Caucasians showed an increased risk to develop pancreatic fat infiltration than the African Americans^[149,150]. More important, PFF may predict the outcome of insulin resistance in African Americans, but didn't show the same accuracy for Hispanics^[151]. However, more research is needed.

The relation with age and low serum lipase activity is probably due to a fatty degeneration of the pancreas, which may be considered paraphysiological^[147,152].

How obesity leads to ectopic fat (EF) accumulation is not clear yet. Some individuals are more susceptible to accumulate EF for Body Mass Index (BMI). Although it may seem paradoxical, it has been suggested that those patients may have an impaired subcutaneous fat storage capacity, which leads to visceral and ectopic fat accumulation. An extreme example of this phenomenon is Lipodystrophy, also known as Berardinelli-Seip syndrome (BSS). Patients with BSS have a scarce subcutaneous fat storage, but a greater amount of visceral and ectopic fat. On the other hand, several studies prove that healthy individuals with high subcutaneous fat content have low levels of visceral and ectopic fat. Subcutaneous fat may even have a protective action regarding ectopic fat accumulation^[153]. In an experimental model, transplantation of normal adipose tissue in the subcutaneous region of lipotrophic mice, removes their excess of EF and insulin resistance^[154].

More efforts have been made to explain ectopic fat accumulation in the liver (NAFLD). It has been hypothesized that insulin resistance facilitates the transport of free fatty acids (FFA) from adipose tissue to the liver, and their storage in hepatocytes. Steatosis occurs when the rate of import and fatty acid synthesis

exceed the rate of export and catabolism^[155].

An interesting finding of a prospective study on 293 patients^[56] is that about 68% of cases with fatty pancreas concurrently had fatty liver, but most subjects (97%) with fatty liver had fatty pancreas. Although the positive predictive value of fatty liver in fatty pancreas was 69.4%, the negative predictive value of fatty liver in normal pancreas was 96.4%. Our group is currently involved in a retrospective CT-based study which preliminary results on 47 patients lead to the same results (Catanzaro R, Cuffari B, Palmucci S *et al*, unpublished). This implies that fatty pancreas could be used as the initial indicator of EF deposition.

As NAFLD and NAFLD are often associated, one could assume that they could share a common pathway^[33,56,57,59,64,75,134-137].

Actually, important information must be taken in account: (1) some studies have found no correlation between NAFLD and NAFLD^[80,156]; (2) hepatic fat is mainly intracellular, while NAFLD is a consequence of adipocytes infiltration^[5,16]; (3) when patients are treated with bariatric surgery, fat loss in the liver and the pancreas seem to be independent, suggesting tissue-specific mobilization of these ectopic fat stores^[77]; and (4) when corrected for BMI, the association between hepatic and pancreatic steatosis can't be found anymore^[33,134,137].

It could be correct to assert that, according with current evidence, the association between NAFLD and NAFLD is a consequence of obesity only. Therefore, hypothesis used to explain hepatic steatosis may not be suitable for NAFLD. However, it is possible that pancreatic and hepatic steatosis affect each other and more research should focus on the different pathways that lead to one or the other condition^[135,157].

In conclusion, the pathogenesis of NAFLD is still unknown, and no satisfactory theories have been proposed yet. Therefore, more research will be needed.

CLINICAL SIGNIFICANCE

T2DM

About 347 million people worldwide have T2DM, and numbers are increasing^[158]. The WHO projects that diabetes will be the seventh leading cause of death in 2030^[159].

Since T2DM is increasing problem worldwide, and pancreatic islets have a key role in the metabolism of glucose, one of the main issues in NAFLD research is whether or not pancreatic steatosis is a risk factor for T2DM.

In vitro and animal studies suggest that pancreatic lipomatosis may contribute to β -cell lipotoxicity and lipoapoptosis, with consequent loss of function^[160-162]. However, data on humans are inconsistent.

Using proton MRS and oral glucose tolerance tests, Tushuizen *et al*^[163] found that pancreatic fat correlated negatively with β -cell function parameters in nondiabetic

subjects. Heni *et al*^[156] found the same association in patients with impaired glucose metabolism and, in a stepwise multivariate regression analysis, pancreatic fat resulted a stronger determinant of impaired insulin secretion than visceral fat. More recently, Della Corte *et al*^[157] found a positive correlation between NAFLD and homeostatic model assessment - insulin resistance (HOMA-IR) in a pediatric population with NAFLD. In contrast with these results, two studies performed using the gold standard hyperglycemic clamp, found no relation between pancreatic fat content and β -cell function in subjects with impaired glucose metabolism^[74,164].

Data about patient with full-blown T2DM are even more challenging. Tushuizen *et al*^[163], found no association between pancreatic fat and β -cell dysfunction in diabetic patients. That may suggest that once diabetes occurs, other factors cause further β -cell impairment. However, they found that diabetic subjects had a significantly higher pancreatic fat content than nondiabetic, association confirmed by Lingvay *et al*^[72].

Saisho *et al*^[30], using computed tomography on 1721 nondiabetic and 165 subjects with T2DM, observed that pancreatic fat was not significantly increased in T2DM.

Wang *et al*^[64], in 2014, studying a cohort of 8097 subjects, found that the fatty pancreas group had an increased risk of diabetes (OR = 1.593) than non-fatty pancreas group ($P < 0.001$).

Finally, a recent study^[165] found a significant higher average fat content in the pancreas of patients with newly diagnosed T2DM compared with healthy controls.

Summarizing, three hypotheses can be made about β -cell dysfunction in pancreatic steatosis: (1) the increased amount of triglycerides in pancreatic β -cells can lead to their dysfunction, probably with a mechanism of lipotoxicity, at least in subjects with an already impaired glucose metabolism; (2) intrapancreatic adipocytes may have a negative paracrine effect on β -cells; and (3) NAFLD and T2DM are just consequences of obesity.

According to current evidence, the majority of the authors support the last theory, but more research should focus on this topic and meta-analysis will be required^[145].

MetS

The MetS is a major and increasing clinical and social issue worldwide, as a result of changing in lifestyle which include high-caloric and high-fat diet and decreasing physical activity. MetS is associated with a 5-fold increase risk of T2DM, 2-fold risk of developing cardiovascular disease, 2- to 4-fold increased risk of stroke, 3- to 4-fold increased risk of myocardial infarction, and 2-fold mortality caused by coronary events^[3].

NAFLD is considered the hepatic manifestation of

MetS. High levels of FFA and insulin resistance are considered key pathogenic factors in the development of fat accumulation in the liver^[3].

There is an increasing evidence of association between NAFLD and all the components of the MetS in animal models and humans^[20,56,59,62,148,166].

Whether pancreatic steatosis is a key organ in the development of the MetS or just a marker of that condition (mediated by general obesity), we believe that the assessment of pancreatic fat infiltration will have an increasing role in the clinical management of the syndrome.

Cardiovascular risk

As discussed, MetS itself is associated with an increased incidence of cardiovascular diseases. Recently, one study^[167] found that pancreatic steatosis may be an independent risk factor on the development of carotid atherosclerosis in non-obese subjects with T2DM. Therefore, it could be a marker of a higher risk of cardiovascular disease, especially in non-obese subjects.

Acute and chronic pancreatitis

Obesity and the MetS are associated with the incidence and severity of acute pancreatitis^[168-171]. Thus now, this association was explained by the fact that both obesity and the MetS are linked with other well-known risk factors for acute pancreatitis, such as gallstones, alcohol abuse, cancer, hypertriglyceridemia, use of medications, moreover, as already pointed out, the MetS may be associated with a pro-inflammatory state which may exacerbate inflammation after the trigger is pulled^[172].

There is speculation that pancreatic steatosis may have a key role in the pathogenesis of pancreatitis in obese patients. In analogy with NAFLD leading to non-alcoholic steato-hepatitis (NASH), the term non-alcoholic steato-pancreatitis (NASP) has been proposed^[48].

The existence of NASP has not been proved yet, but there is biological plausibility, and evidence is increasing. Adipocytes may generate a local and systemic pro-inflammatory state by producing pro-inflammatory adipokines and cytokines, such as leptin, interleukin 1 β and tumor necrosis factor, or toxic fatty acids that may make the pancreas more susceptible to inflammation^[28,157,173].

Pokhrel *et al.*^[174] found that increased pancreatic fat on MRI was not an independent predictor of post-ERCP pancreatitis, however, data is scarce and more research will be required.

An excellent review by Acharya *et al.*^[175] points out that intra-pancreatic fat have a role in the severity of acute pancreatitis: lipases released by acinar cells after the first injury, cause a local and systemic lipolysis, which results in increasing of FFAs, especially unsaturated fatty acids (UFAs). The spread of UFAs in

the pancreatic parenchyma has a direct toxic effect on acinar cells (lipolytic flux), causing acinar necrosis. In post-mortem studies, more severe necrosis of the parenchyma occurred closer to necrotizing fat (peri-fat acinar necrosis: PFAN)^[143,176]. In 2011, Smits and van Geenen^[9] published preliminary results of a CT-based study that show a significant relationship between pancreatic steatosis and severity of pancreatitis ($P < 0.03$). The use of lipases inhibitors (Orlistat) to prevent conversion of mild into acute pancreatitis may be therefore justified and studies are evaluating its efficacy, with encouraging results^[177].

As already discussed, RAP can cause chronic pancreatitis and morphological changes, which include fatty replacement. However, no evidence exists that NAFLD can cause chronic pancreatitis.

Pancreatic fibrosis

Chronic liver inflammation as seen in NASH, leads to necrosis of hepatocytes and liver fibrosis. Theoretically, in the pancreas, NASP could determinate the same changes, but data are inconsistent.

Matsuda *et al.*^[26], found that in Zucker Diabetic Fatty rats fed with a chronic high-fat diet, fat accumulates in pancreatic acinar cells, and this fatty change seems to be related to subsequent pancreatic fibrosis and acinar cell injury. However, in Ossabaw swines, another animal model for the MetS, pancreatic steatosis was not associated with other histological abnormalities^[20].

In humans, patients with chronic pancreatitis have both an increased amount of pancreatic fat and fibrosis, however, van Geenen *et al.*^[35] found no relationship between pancreatic fibrosis and NAFLD ($P = 0.916$) in a study over 900 autopsies, and Mathur *et al.*^[31] found that pancreatic fat correlated even negatively with fibrosis ($P < 0.001$).

Therefore, according to current evidence, fatty replacement and pancreatic fibrosis seem to be both independent consequences of chronic inflammation in patients with chronic pancreatitis.

Pancreatic cancer

Several studies show that obesity is one of the leading risk factors for pancreatic cancer (PC)^[178-184]. However, the pathophysiological pattern of this association is not clear yet. Several mechanisms have been discussed^[172]: Evidence exists that the increase of certain hormones in obese patients, such as insulin, adipokines and resistin and systemic oxidative stress may have a role in the development of pancreatic adenocarcinoma^[172,185-188].

NAFLD has been independently associated with an increased risk to develop PC^[38]. A recent study^[40] observed a correlation between pancreatic intraepithelial neoplasia (PanIN) and extra- ($P < 0.01$) and intralobular ($P < 0.0001$) pancreatic fat. No clear metabolic pathways have been identified to explain this association, but speculation is possible.

The increased numbers of adipocytes in NAFPD could promote cancerogenesis indirectly by NASP, as occurs in the liver^[9].

Persistent organic pollutants (POPs) are lipophilic toxics able to bio-accumulate in fatty rich tissues of animals, especially those higher in the food chain, humans included. Evidence exists that accumulation of POPs in adipose tissue may be associated with PC occurrence, and it is possible that a higher concentration of pancreatic fat, and consequently of POPs, can partially explain the linkage between NAFPD and PC^[172].

Along with an increased risk to develop PC, patients with increased pancreatic fat have a poorer outcome than those who develop cancer in a lean pancreas. In particular, NAFPD promotes dissemination and lethality of PC^[32,67]. Mathur *et al*^[32] suggested that "pancreatic steatosis alters the tumor microenvironment, enhances tumor spread, and contributes to the early demise of patients with pancreatic adenocarcinoma".

In addition, pancreaticoduodenectomy in patients with fatty pancreas is associated with an increased risk of intraoperative blood loss and post-operative pancreatic fistula, which contributes with the poor outcome in those patients^[31,34,37,50,68,71,189-191]. Therefore, assessment of pancreatic steatosis by preoperative CT or MRI or intraoperative histological evaluation on the frozen sections may have a role, in the future, in the prognostic evaluation of patients with PC^[37,67,68,71,189,190].

Pancreatic transplant

The first pancreatic transplant was performed in 1966 in the United States^[192]. It is a very effective and yet the only definitive option for curing insulin-dependent diabetics. However, the spreading of this technique is restricted by the significant rate of surgical complications resulting in graft failure/loss and recipient morbidity and mortality.

Increasing obesity and age, of both recipient and donor, increase the risk of technical complications like graft pancreatitis, graft thrombosis, intra-abdominal infections, gangrene and pancreatic fistula. However, if the transplantation is successful, there is not an increased risk of allograft failure^[193,194]. Older patients with higher BMI are more likely to have steatosis, and that could explain the association.

Certain transplant surgeons do not use pancreas that are infiltrated by fat on inspection, since the procedure is technically more difficult when using a fatty pancreas, but a more objective measurement could avoid to waste organs that would be suitable for transplant. Verma *et al*^[11] propose the IDEAL scanning on the sole organ as a possible solution. Furthermore, "defatting" of the organ is possible and a successful case has been reported in 2004^[195].

Pancreatic hyperenzimemia

In 1996, Gullo *et al*^[196] first reported a benign

syndrome characterized by increased levels of serum amylase, pancreatic isoamylase, lipase and trypsin. This condition is nowadays called Gullo's syndrome and has been better characterized^[196,197].

Cavallini *et al*^[198], in a US based study, found that HP was related with hyperamylasemia. However, Gullo *et al*^[54] found no correlation in a MRI based study. More recently, Wu *et al*^[62] found that amylase levels in patients with pancreatic lipomatosis were even lower than controls.

Exocrine function

In theory, pancreatic steatosis can lead to EPI with different mechanisms: (1) fat droplet accumulation in acinar cells and consequent lipotoxicity; (2) negative paracrine effect mediated by adipocytes; and (3) death of acinar cells as cause of both EPI and fatty replacement.

However, the exocrine function in patients with NAFPD has never been extensively investigated. Therefore, data are mainly based on case reports of extreme cases of complete fatty replacement of pancreas with fatty tissue^[36,52,199-201].

In one recent study^[202], fecal level of pancreatic elastase (PE-1) was evaluated in patients with T2DM. EPI was more frequent in patients with high HbA1c, but did not correlate with pancreatic steatosis.

THERAPY

Since NAFPD has been only recently extensively studied and its clinical significance is not clear, no clinical trials have validated any medications yet. Anyway, evidence exist that it is reversible.

Pancreatic fat can be reduced by weight loss, with or without bariatric surgery, and that is associated with improvement of insulin sensibility^[203].

Moreover, specific treatment showed efficacy *in vitro* or on murine models: (1) Troglitazone^[204]; (2) combination of Telmisartan and Sitagliptin^[21]; and (3) Berberine and Cinnamic Acid, as components in Jiaotai Pill, a traditional Chinese medication^[27,205].

We hope that increasing evidence on the clinical significance of pancreatic steatosis will support further research.

CONCLUSION

Pancreatic steatosis, which comprehends fatty replacement and fatty infiltration of the pancreas, is a very common condition, easily diagnosable but often neglected by physicians and researchers.

Its clinical significance ranges widely in Internal Medicine and Surgery, and more correlations may be found in future. Nevertheless, evidence is not exhaustive and the pathophysiology is yet unknown.

We believe that, according to current literature, pancreatic steatosis should have stronger consideration

in clinical practice, in particular: (1) as an early marker of ectopic fat accumulation and insulin resistance in the MetS; (2) in the differential diagnosis with pancreatic fibrosis, especially when the pancreas is observed with US or EUS; (3) as a prognostic and/or predictive marker for acute pancreatitis and PC; (4) in pre-operative evaluation before pancreaticoduodenectomy or pancreatic transplant; and (5) as a possible cause of unexplained exocrine pancreatic insufficiency.

More research, in future, should focus on the clinical consequences of pancreatic steatosis, in order to understand its impact on human health, its pathophysiology and eventually support clinical trials.

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Tolerance in liver transplantation: Biomarkers and clinical relevance

Alberto Baroja-Mazo, Beatriz Revilla-Nuin, Pascual Parrilla, Laura Martínez-Alarcón, Pablo Ramírez, José Antonio Pons

Alberto Baroja-Mazo, Beatriz Revilla-Nuin, Pascual Parrilla, Laura Martínez-Alarcón, Pablo Ramírez, José Antonio Pons, Biomedical Research Institute of Murcia (IMIB-Arrixaca-UMU), 30120 Murcia, Spain

Pascual Parrilla, Pablo Ramírez, José Antonio Pons, Division of Gastroenterology and Hepatology and Liver Transplant Unit, University Hospital Virgen de la Arrixaca, 30120 Murcia, Spain

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Correspondence to: José Antonio Pons, MD, PhD, Division of Gastroenterology and Hepatology and Liver Transplant Unit, University Hospital Virgen de la Arrixaca, Ctra. Madrid-Cartagena, s/n, 30120 Murcia, Spain. joseapons.imib.arrixaca@gmail.com
Telephone: +34-968-369500
Fax: +34-968-369776

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Abstract

Transplantation is the optimal treatment for end-stage organ failure, and modern immunosuppression has allowed important progress in short-term outcomes. However, immunosuppression poorly influences chronic rejection and elicits chronic toxicity in current clinical practice. Thus, a major goal in transplantation is to understand and induce tolerance. It is well established that human regulatory T cells expressing the transcription factor FoxP3 play important roles in the maintenance of immunological self-tolerance and immune homeostasis. The major regulatory T cell subsets and mechanisms of expansion that are critical for induction and long-term maintenance of graft tolerance and survival are being actively investigated. Likewise, other immune cells, such as dendritic cells, monocyte/macrophages or natural killer cells, have been described as part of the process known as "operational tolerance". However, translation of these results towards clinical practice needs solid tools to identify accurately and reliably patients who are going to be tolerant. In this way, a plethora of genetic and cellular biomarkers is raising and being validated worldwide in large multi-center clinical trials. Few of the studies performed so far have provided a detailed analysis of the impact of immunosuppression withdrawal on pre-existing complications derived from the long-term administration of immunosuppressive drugs and the side effects associated with them. The future of liver transplantation is aimed to develop new therapies which increase the actual low tolerant vs non-tolerant recipients ratio.

Key words: Liver transplantation; Operational tolerance;

Regulatory T cells; Dendritic cells; Biomarkers

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Core tip: Nowadays, the major goal in transplantation is to understand and induce tolerance. Although a plethora of genetic and cellular biomarkers is raising and being validated worldwide in large multi-center clinical trials, little is known about the impact of immunosuppression withdrawal on pre-existing complications derived from the long-term administration of immunosuppressive drugs and the side effects associated with them. The future of liver transplantation is aimed to develop new therapies which increase the actual low tolerant *vs* non-tolerant recipients ratio.

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INTRODUCTION

In 1953, Peter Medawar and his colleagues described in their key paper^[1] that “acquired tolerance is due to a specific failure of the host’s immunological response”. Following on from this pioneering work of Medawar and his colleagues more than 50 years ago, extensive data obtained from rodents and large animal experimental transplantation models have led to a better understanding of the mechanisms leading to graft rejection and transplantation tolerance. In clinical transplantations since 1995, there has been increasing evidence to demonstrate that liver transplant recipients who cease to take immunosuppressive drugs maintain allograft function, suggesting that tolerance is already present^[2,3]. Graft acceptance in the presence of significantly reduced immunosuppression (IS) requirements is referred to as “prope tolerance” or “minimal IS tolerance”^[4]. In the clinical setting, “operational tolerance” (OT) is defined as the absence of acute and chronic rejection, and graft survival with normal function and histology in an IS-free, fully immunocompetent host, usually as an end result of a successful attempt at IS withdrawal^[5]. Although complete immunosuppressive drug withdrawal has been rarely performed in an intentional manner, accumulated experiences from selected institutions indicate that this strategy is feasible in 20% of liver transplant recipients^[6]. The achievement of immune tolerance to an allergenic donor has been a field of intense research over the last decades, fuelled by a critical need to avoid IS-related side effects (particularly nephrotoxicity, cancer, and cardiovascular events).

Unfortunately, true immunologic tolerance has been difficult to achieve, in part, because allergenic engraftment is not a naturally occurring phenomenon and graft rejection is the most powerful and diverse immunologic response known. In recent years, the main endpoint of immunosuppressive therapy has shifted from the prevention of acute rejection toward the preservation of long-term graft function^[7,8]. For instance, Foxp3-expressing regulatory T cells (Treg) critically prevent the occurrence of autoimmunity and suppress various immune responses. Some of the studies indicated that higher presence of Tregs correlated with better transplant outcomes, but some showed Tregs do not affect graft function and survival. The conclusion of each study might be limited to their study design or small sample size. Here, we review the development and function of Tregs, and how these cells are used to facilitate the induction of transplantation tolerance. Moreover, while dendritic cells (DC) are highly efficient antigen presenting cells (APC) for exerting allergenic immune responses, DC are also involved in establishing immune tolerance by deleting T cell clones or inducing Tregs^[9], and we describe attempts of using tolerogenic DC as a therapeutic strategy to promote transplant tolerance. Likewise, we detail the implication of other cells in both the innate and adaptive immune system to diminish allergenic response.

In the other hand, development of new immunosuppressive drugs treating to minimize the adverse events while maintaining immunosuppressive efficacy are raising. The inhibitors of mechanistic target of rapamycin (mTOR), such as rapamycin and its derivate everolimus, are powerful non nephrotoxic agents with a different mechanism of action than calcineurin inhibitors (CNI), which blocking growth-factor-mediated cell proliferation in the cellular response to alloantigen^[10,11], and could maintain an adequate level of IS while concomitantly promoting an immunologic profile which could favor tolerance to the graft.

Last but not least, a review of different attempts to establish a biomarker signature which define liver transplant recipients who are candidates to be subjected to a weaning protocol will be addressed in the last part of this overview.

REGULATORY T CELLS IN TRANSPLANTATION AND TOLERANCE

Regulatory cells are defined by their functional ability to suppress immune responses. In 1970, Gershon and Kondomade the seminal finding that T cells not only augmented but also dampened immune responses and that this down-regulation was mediated by T cells that were different from Th cells^[12]. The term regulatory or suppressor cells was reintroduced in 1995 based on studies with mice thymectomized in the neonatal period that developed a fatal autoimmune disease^[13].

They identified the CD25 molecule [the interleukin (IL)-2 receptor α -chain] as a Treg surface molecule candidate because CD25+ T cells, which constituted 5%-10% of peripheral CD4+ T cells (and less than 1% of peripheral CD8+ T cells) in normal naive mice, were confined in the CD25^{high} and CD45RB^{low} fraction of CD4+ T cells. The identification of the forkhead box P3 (FoxP3) as a key transcriptional factor in Tregs has enabled us to determine a number of immunological characteristics of natural Tregs, including their function, stability and differentiation^[14-16]. FoxP3 was initially identified as the responsible gene for an X-linked recessive inflammatory disease in scurfy mutant mice and then for the fatal autoimmune/inflammatory disease, immune dysregulation, polyendocrinopathy, enteropathy or X-linked syndrome (IPEX) in humans^[17]. The indispensable role of FoxP3 for the control of these autoimmune and inflammatory disorders underlines the crucial importance of naturally arising FoxP3+CD4+ Tregs for self-tolerance and immune homeostasis^[18].

Although other subsets of cells with regulatory functions have been described, CD4+CD25+FOXP3+ regulatory T-cells are the classic example. These cells are defined by FOXP3 expression, but they are not a homogeneous subset of cells. Regulatory T-cells can be subdivided into naturally occurring regulatory T-cells, which develop in the thymus, and adaptive or induced regulatory T-cells, which are converted from CD4+CD25- T-cells into cells with a characteristic molecular profile in peripheral blood^[19]. Stable expression of FoxP3 in naturally occurring Tregs requires DNA methylation-based regulation^[20]. Demethylation at a highly conserved region within the human *FoxP3* gene (Tregs-specific demethylated region, TSDR) was found to be restricted to Tregs when tested against all major peripheral blood cell types and a selection of non blood cells^[21]. In addition to the high specificity for Tregs, it was also observed that FoxP3 TSDR demethylation occurred only in natural Tregs, but not in recently activated effector T cells transiently expressing FoxP3^[22]. Some authors found that FoxP3 was fully demethylated in nTregs, partially demethylated in TGF- β -polarized iTregs and methylated in naive and Th cells^[21,23]. TSDR demethylation does not act as an on/off switch, but corresponds with stability of FoxP3 expression determined during the development in the thymus for naturally occurring Tregs. This data indicated that epigenetic modifications in the FoxP3 TSDR serve as a valuable marker for the identification of nTregs with a stable Tregs phenotype^[24].

Although the exact mechanism of action of regulatory T-cells is still under debate, studies involving animal models have provided some insight. Such studies have shown, for example, that regulatory T-cells use several mechanisms to inhibit effector T-cells: modulation of APC function; metabolic disruption such as IL-2 deprivation or adenosine secretion; direct cytotoxicity toward effector T-cells; and secretion of

inhibitory cytokines such as IL-10, IL-35 or TGF- β ^[25]. In the absence of regulatory T-cells, effector T-cells recognize alloantigens presented in the context of MHC molecules by APCs directly (donor APCs) or indirectly (recipient APCs). After T cells are activated by the binding of alloantigen-MHC to the TCR, and of CD80/86 to CD28, IL-2 is secreted by the effector T-cells, and through autocrine mechanisms leads to further T-cell activation, and proliferation and differentiation, ultimately causing allograft rejection. Regulatory T-cells inhibit APC function by down-regulating the expression of co-stimulatory molecules on APCs and by inducing APCs to produce immunoregulatory enzymes, such as indoleamine dioxygenase, that alter the metabolic microenvironment and depleting essential amino acids. In addition, the interaction between CD80/86 expressed by APCs and CTLA-4 expressed by regulatory T-cells is essential in mediating allograft tolerance. Once regulatory T-cells are activated, they secrete TGF- β , IL-10 and adenosine, which inhibit effector T-cells and render them unresponsive (anergy) or tolerant towards the graft. Collectively, these mechanisms protect the graft^[25], such as Tregs are considered to be critical for the induction of transplant tolerance. Transplantation of MHC histoincompatible tissues elicits a strong, cytopathic, T cell-dependent immune response to donor tissues. In this T cell-dependent pathway to rejection, donor alloantigens are processed by donor (direct pathway of allorecognition) or recipient (indirect pathway of allorecognition) specialized APCs. The characteristics of the inflammatory environment in which donor-reactive CD4+ T cells recognize donor antigens determine the lineage commitment of these cells. Thus, depending on the cytokines present when antigen activation occurs, naïve CD4+ Th cells can acquire a variety of cytopathic and/or immunoregulatory phenotypes^[26]. In the absence of proinflammatory cytokines, transforming growth factor TGF- β induces expression of FoxP3 and differentiation of CD4+ T cells into Tregs. In contrast, expression of TGF- β with IL-6 or IL-21 prevents development of the transplant-protective Tregs; instead, the antigen-reactive CD4+ T cells become IL-17-producing T cells (Th17), which are highly cytopathic^[27-29]. Recent discoveries also revealed that, instead of being terminally differentiated, Th17 and Tregs have remarkable plasticity and are closely interlinked^[30]. Thus, Tregs can differentiate into IL-17-producing cells in the presence of IL-2 and IL-1- β whereas in the presence of IL-27, Th17-producing cells also produce IL-10, an immunosuppressive cytokine that prevents them from functioning as destructive effector cells^[31,32]. The current paradigm is that the outcome of transplant recipients, rejection or graft acceptance, is determined by the relative balance between cytopathic Th1 and Th17 CD4+ T cells vs rejection-blocking, cytoprotective Tregs; this balance depends on the level of inflammation in the microenvironment in which T-cell activation takes place^[33].

Tregs have been used as a diagnostic tool in organ transplantation, and Tregs counts have been measured in blood, biopsy and urine samples after transplantation in many studies^[34,35]. Although not unanimous, some studies have suggested that Tregs is associated with better outcome and can also serve as an immune marker to predict the individual risk of rejection and identify tolerant patients^[36-41].

In liver transplantation (LT), several trials have been conducted to assess the feasibility of purposely discontinuing all immunosuppressive drugs under medical supervision^[33]. Three studies reported the relationship between Tregs and transplant tolerance in LT^[38,40,42,43] and demonstrated that Tregs content and function werenot lower in tolerance groups than chronic rejection group, stable group and control group, which suggested that Tregs may be associated with transplant tolerance. This Tregs increment was reported in retrospective studies where long-term operationally tolerant patients were compared with immunosuppressed patients. In the context of human LT, the dynamics of Tregs have not been extensively studied and may afford a means of identifying transplant recipients with a predilection to developing tolerance. Therefore, the immune process that occurs during the weaning off the IS was not analyzed. Our group carried out a prospective study to investigate the dynamic profile of the Tregs population in liver transplant patients during IS withdrawal and whether this profile could aid identification of patients who develop operational tolerance^[44]. In this study the first evidence was provided to demonstrate that the increase of CD4+CD25high T cells and FoxP3 transcripts was associated with operational tolerance in liver transplanted patients during IS withdrawal.

Nowadays, Tregs are used as a cellular therapy for controlling rejection. *In vitro* and *in vivo* experimental models have demonstrated that production of Tregs in the periphery by FoxP3 transfection in naïve T cells can lead to tolerance induction and graft acceptance^[45,46]. Nadig *et al*^[47] have demonstrated that *ex vivo* expanded CD25hiCD4+ and CD127loCD25+CD4+ Tregs are very effective at inhibiting vasculopathy, with CD127loCD25+CD4+ cells being five times more efficient than T cells selected on the basis of high levels of CD25 expression prior to *ex vivo* expansion. These experimental data gave support to the potential use of Tregs in clinical transplantation. Many strategies exist for the *ex vivo* generation and/or expansion of Tregs^[35,48-50]. Currently, three main approaches are being explored for Tregs expansion in the perspective of therapeutic protocols: *ex vivo* nTregs expansion, *ex vivo* conversion of naïve T cells to iTregs and *in vivo* expansion of nTregs and/or induction of iTregs^[51-53]. Besides co stimulatory blockade, T-cell depletion induction therapies (e.g., anti-CD3, anti-CD52 monoclonal antibodies or polyclonal anti-thymocyte globulins) are used in clinical solid organ transplant (SOT) to prevent acute rejection. These

therapies induce profound and durable (weeks to months) reduction of circulating lymphocytes capable of mounting an alloresponse. Recent data suggests that T-cell depletion protocols allow preferential expansion of Tregs once lymphocytes gradually repopulate the host, thus skewing the Tregs/effector T cells ratio towards tolerance^[54]. The “first-in-man” studies with expanded nTregs were carried out in patients who developed GVHD following bone marrow transplantation^[55-58]. However, the use of antigen-specific Tregs at the time of transplantation may be limited if the donor is cadaveric, *i.e.*, not known in advance, as time is required to generate and expand *ex vivo* donor-specific Tregs. In the contrary, if a living donor is available (HSCT, kidney, LT), recipient (or donor in the case of HSCT) T cells could be isolated in advance and manipulated *ex vivo* in the presence of donor-derived APC or peptides. Efficient isolation, expansion and cryopreservation strategies that comply with good manufacturing practice (GMP) guidelines are prerequisites for the clinical application of human CD4+CD25+CD127low FoxP3+ Tregs. Although the existence of Tregs is indisputable, using them for therapeutic purposes has not been straightforward; in fact the local microenvironment in which Tregs reside can have a considerable influence on their functional status^[59]. In addition, one of the obstacles in the implementation of clinical protocols using Tregs is their low frequency in the peripheral blood leading to the need for *ex vivo* multiplication of the cells prior to their use *in vivo*^[60]. While the transfer of Tregs prolonged allograft survival, it was not sufficient to induce robust tolerance on its own. This highlights the need for adjuvant immunomodulatory therapies to suppress strong immune activation and overcome the rapidly expanding pool of alloreactive T cells early after transplantation. Thus, the *in vivo* homeostasis, lifespan and stability of nTregs and iTregs need to be clarified before clinical trials on Tregs transfer can be considered.

DCs IN TOLERANCE

DCs act as surveillance for the immune system, sampling self and exogenous antigens in the peripheral tissues and presenting them to T cells in lymphoid organs. So, APCs serve as a bridge between antigens and lymphocytes. Likewise, DCs providing additional costimulatory signals and cytokines to stimulate the immune response. Functions of DCs stem from their high expression of surface major histocompatibility complexes (MHC) class I and II, costimulatory molecules and adhesion molecules^[61]. Apart from their immunogenic roles, the influence of DCs on the immune system can also be tolerogenic or inhibitory in nature. DCs have been shown to be critical in maintaining central and peripheral tolerance through immune deviation, induction of T cell anergy, promotion of T cell apoptosis and induction of Tregs.

The importance of DCs in transplant rejection was highlighted by the finding that graft rejection was related to the migration of immunogenic passenger DCs into recipient lymphoid tissues, instigating rejection^[62]. Donor derived DCs from allografts have the ability to directly migrate to recipient secondary lymphoid tissues to initiate immune responses^[63], referred as direct pathway of allorecognition. Recipient DCs can be equally implicated in transplant rejection, through indirect pathway of allorecognition, as evidenced by the fact that skin allografts from MHC class II^{-/-} donors onto MHC class I^{-/-} recipients were still rejected^[64]. However, more recently the view on recipient DCs as being solely potent stimulators of T cells has changed, based on evidence demonstrating the role of DCs in central and peripheral tolerance^[65-67]. The overall tolerogenic or pathogenic capacity of DCs is dictated by: (1) the DC subset involved; (2) the maturation status; and (3) the microenvironment^[61,68,69]. DC subsets differ on surface marker expression, tissue distribution and function. Human DCs subsets display a vast array of subsets: myeloid, plasmacytoid or follicular DCs and Langerhan's cells^[70,71]. It is known that production by immature DCs of indoleamine 2,3-dioxygenase (IDO), which catabolizes the essential amino acid L-tryptophan, evokes an amino acid deprivation, inhibiting antigen specific T cell proliferation while promoting Tregs development and tolerance^[72,73]. The local microenvironment can have a significant impact on the development of DCs. Certain locations promote greater numbers of tolerizing DCs than others. Liver-derived DC progenitors were more suppressive than bone marrow derived DCs^[74,75]. *In vivo* development of DCs is driven by hematopoietic growth factors c-Kit ligand and Fms-like tyrosine kinase 3 (FLT-3)^[76-78], and production of granulocyte and monocyte colony stimulatory factors (GM-CSF) by activated T cells serves as maturation signal for DCs. Fully mature DCs produce proinflammatory cytokines and upregulate costimulatory molecules^[79]. In contrast, immature DCs produce tolerogenic cytokine IL-10 and lack costimulatory signals for T cell activation.

Although the major mechanism of immune tolerance occurs in the thymus, DCs induce peripheral tolerance through: (1) deletion of alloantigen specific T cells; (2) induction of T cell anergy; (3) immune deviation; and (4) generation of regulatory T cells^[9]. Transplantation of allogenic organs generates high frequencies of alloreactive T cells, and deletion of donor-reactive T cells is critical in the induction of transplant tolerance. Elimination of donor-reactive T cells by DCs could be carried out through either inhibitory signaling or production of apoptotic factors^[75,80,81]. The mechanisms through which immature DCs induce T cell anergy are not understood but thought to involve IL-10, directly through IL-10 receptor signaling or dependent on inducible T-cell costimulator (ICOS) signaling^[82,83]. Another reason for considering tolerogenic DCs for tolerance therapies in transplantation is their ability to skew the cytokine profile in the direction of a Th2

phenotype^[84]. DCs induce contrasting states of immunity or tolerance based on their maturation and subset. Both *in vivo* and *in vitro* evidence support the central role of IL-12 in the polarization of Th1 lymphocytes. Levels of IL-12 fluctuate during the different stages of DC development, therefore, DCs can differ in their immunogenicity depending on their maturation state. Tolerogenic DCs can in part mediate Tregs suppressive functions by promoting their development, principally by immature DCs^[85]. Tolerogenic DCs may not only be involved in the induction of Tregs but may also play a role in the activation and maintenance of their suppressor functions. IDO has been shown to skew naive CD4+ T cell development towards the Treg lineage^[86], and it was dependent on cell-cell contact mediated mechanisms^[87]. Interestingly, Tregs may also promote the development of tolerogenic DCs from DC progenitors^[88].

In this way, tolerogenic DCs are tempting from a clinical perspective because of their low capacity for T cell stimulatory functions and high capacity for inducing tolerogenicity. Tolerogenic DCs differ phenotypically and functionally from their mature DC counterparts. Downregulation of MHC class II^[89] and costimulatory molecules as CD40, CD80, CD86 or CD83^[90,91] or upregulation of inhibitory factors as B7-H1 or ICOS ligand^[92] and death inducing ligands as FASL or TRAIL^[93,94] on the surface of tolerogenic DCs compromises their ability to present antigen and activate T cells. In addition to contact dependent mechanisms of inhibition, tolerogenic DCs secrete effector molecules and regulatory cytokines (nitric oxide, heme-oxygenase-1, IL-10), thereby extending their suppressive effects^[95-97].

In vitro propagation of DCs is necessary to generate the number of DCs required for therapeutic applications, due to DCs constituting a small fraction of leukocytes. In this way, several techniques have been carried out to manipulating DCs for therapeutic purposes. Maturation with GM-CSF^[92] and tolerogenic cytokines, blocking the costimulatory pathway^[74,98], using immunosuppressive drugs as rapamycin^[99] or by genetic engineering^[100,101].

OTHER CELLS: B CELLS, MACROPHAGES AND NATURAL KILLER CELLS

B cells not only serve as an effector component of an immune response by generating antibodies, but they also present antigens to T cells and release immune cytokines. They may help to generate and expand Tregs as well as diminish antigen-specific T cell responses^[102,103]. B cells also produce cytokines under inflammatory conditions. In particular, B cells produce large amounts of the immunosuppressive cytokine IL-10, which inhibits and reverses the progression of inflammation. Both CD5+ B1 cells and conventional B cells have been reported to produce IL-10^[104]. These findings suggest that B cells may be critical regulators

in the process of tolerance induction. Clinical trials in renal transplantation revealed a significant increase of total B cell numbers and naive B cells in tolerant recipients^[103,105]. Moreover, tolerant patients also had enhanced expression of B cell differentiation and activation genes such as TCL1A or VH4-34. It remains unknown whether the elevation of B cell numbers was a consequence of transplantation tolerance or whether the B cells were involved in promoting tolerance.

Other innate cell types exhibit similar features in tolerance induction. In certain settings, monocyte/macrophage can exert potent anti-inflammatory and immunosuppressive effects that help maintain peripheral tolerance^[106]. The alternative activated M2 macrophages are capable of secreting anti-inflammatory cytokines, such as IL-10 and TGF- β that are involved in tapering immune responses and resolution of graft inflammation. In fact, some studies demonstrate that adoptive transfer of M2 macrophages can ameliorate the induction of experimental autoimmune encephalitis and prevent autoimmune colitis by inducing and expanding Tregs^[107]. Additionally, adoptive transfer of donor-derived M2 macrophages in a cohort of human kidney transplant recipients allowed for significant reduction in the use of immunosuppressive drugs. Similarly, natural killer (NK) cells also employ different mechanisms to promote transplant tolerance. NK cells, guided by "missing self recognition", can eliminate graft-derived allergenic DCs, thus reducing T cell priming by the direct pathway of antigen presentation^[108]. Killing of donor cells by NKs favours the indirect antigen presentation, which is implicated in tolerance induction. Also, some NK cells exhibit regulatory function through IL-10 dependent mechanisms and contribute to tolerance induction by tipping the balance towards regulation^[109].

The striking dichotomy of innate immune cells in transplant settings (rejection vs tolerance) is most likely context dependent, representing opposite outcomes of the immune response to allotransplants. Along this line, NK cells can be tolerogenic, and further NK maturation by IL-15 mediates rejection^[110]. Likewise, M1 macrophages are pro-inflammatory and M2 macrophages are immunosuppressive. This context-dependent function of innate pathways and context-dependent regulation of innate immune cells constitute a major challenge in manipulating immune responses to allotransplants.

IMMUNOSUPPRESSIVE DRUGS IN TRANSPLANTATION TOLERANCE: RAPAMYCIN

CNI, such as tacrolimus and cyclosporine A, have become the principal immunosuppressive drug in solid organ transplantation^[111]. Their use resulted in lower rejection rates and improved short-term allograft survival rates, although long-term improvements

have been more difficult to achieve. The main reason is that prolonged CNI exposure is associated with nephrotoxicity^[112], neurotoxicity^[113], risk for cancer^[114], metabolic complications^[115], and hypertension^[116]. Reducing CNI exposure is the main goal to lower these adverse events, maintaining immunosuppressive efficacy. The inhibitor of mTOR, such as rapamycin and its derivate everolimus, are powerful non nephrotoxic agents with a different mechanism of action than CNI. Meanwhile CNI block the production of proinflammatory cytokines leading to inhibition of T-cell activation, rapamycin reduce T-cell activation later in the cell cycle by blocking growth-factor-mediated cell proliferation in the cellular response to alloantigen^[10,11]. The distinct mechanism of action and favorable nephrotoxicity profile has led to rapamycin-containing regimens being developed with the aim of minimizing, eliminating, or avoiding exposure to CNI. mTOR is a protein kinase involved in the signal 3 pathway of lymphocyte activation^[117]. More specifically, mTOR belongs to the PI3K pathway, which is involved in several fundamental cellular functions such as cell growth, proliferation, and survival. The mTOR protein interacts with several proteins to form two distinct complexes: mTOR complex 1 (mTORC1) and 2 (mTORC2)^[118]. Rapamycin interact with and inhibits mTOR, but only when it is part of mTORC1 and not mTORC2^[118].

Rapamycin mediates immunosuppressive effects through multiple immune cell types and processes. Inhibition of mTOR by rapamycin suppresses the immune response by preventing cell cycle progression from G1 to S phase, thereby blocking proliferation^[119]. Likewise, rapamycin can promote T-cell anergy independently of the inhibition of proliferation even in the presence of TCR activation and co-stimulation by CD28 and IL-2^[120,121]. Other important functions of rapamycin in the immune system are related to dendritic cells. Rapamycin inhibits the ability of dendritic cells to endocytose antigens, to express MHC class II molecules and to express co-stimulatory molecules^[122,123], thereby preventing these cells from fully maturing into APCs that can strongly stimulate T-cells. Furthermore, immature dendritic cells promote the expansion of regulatory T-cells thus promoting tolerance to the graft^[124]. This is explained by the observation that the JAK/STAT signaling pathway is induced preferentially in regulatory T-cells, whereas the PI3K/AKT/mTOR signaling pathway is reduced relative to conventional T-cells^[125]. In addition, rapamycin induces the expression of high levels of the anti-apoptotic proteins Bcl-2 and Bcl-xL in regulatory T-cells; however, it downregulates the expression of such proteins in conventional T-cells^[126].

Many studies have confirmed the beneficial effects of rapamycin or everolimus on regulatory T-cell biology^[127-129]. Patients treated with rapamycin before an allergenic corneal transplant showed an increased percentage of regulatory T-cells after transplantation^[130], these changes were associated with inhibition

Table 1 Biomarkers of tolerance in liver transplantation

Biomarker	Description	Study before or during IS withdrawal	Ref.
<i>Dendritic cells</i>			
pCD/mCD ratio	Tolerant patients have elevated pDC/mDC ratio. No differences between tolerant patients <i>vs</i> healthy controls	No	[134,150]
mDCs/pCD ratio	Elevated mDC/pCD associated with late rejection	No	[151]
PD-L1/CD86 ratio	Elevated PD-L1/CD86 expression on DCs in tolerant patients	No	[152]
DC HLA-G expression	Elevated on mDC	No	[153]
<i>T cells</i>			
Regulatory T cells	Increase of peripheral CD4+CD25high cells and RNA FoxP3 over time during weaning Increase in T regs in liver biopsy of tolerant patients	Yes	[40,43,44,133]
Natural killer	Increase in Tolerant patients	Yes	[154]
<i>Soluble factors</i>			
Serum HLA-G	Normal or elevated serum HLA-G levels associated to normal liver	No	[155-157]
Anti-donor antibodies	Absent in tolerant patients	No	[150]
<i>Cell proliferation</i>			
Phytohemagglutinin SI	SI < 20 and > 10 yr since LT 100% tolerance	Yes	[158]
<i>Genetic profile</i>			
Cytokine gene polymorphism	Low TNF-alpha and high/intermediate IL-10 production in OT	No	[159]
Gene transcripts	Enriched from NK, CD4+CD25+ FoxP3+, $\gamma\delta$ TCR+ and δ 1TCR+	No	[42,43,160]
Genes related to iron homeostasis in liver graft	Enriched in tolerant patients (CDHR2, MIF, PEBP1, SOCS1, TRF)	Yes	[140]

DC: Dendritic cell; mDC: Monocytoid dendritic cell; LT: Liver transplantation; pDC: plasmacytoid dendritic cell; SI: Stimulation index.

of graft rejection. In another^[128], patients treated with everolimus maintained constant levels of CD4+ T-cells during the treatment, but patients treated with CNi showed a decrease of these cells. Moreover, patients treated with everolimus had higher percentage of total CD4+ and naïve CD4 T-cells than those treated with CNi. With patients receiving IS, maintaining a pool of naïve T-cells is of great importance to protect against new infective agents. In addition, compared with cyclosporine A-treated patients, everolimus-treated patients had more regulatory T-cells and regulatory T-cells expressing CXCR3, a chemokine receptor that is responsible for the migration of T-cells to inflamed tissue such as the transplanted liver. Thus, everolimus seems to be more effective in preventing rejection by allowing regulatory T-cells to exert an effect *in situ*. Cyclosporine A-treated patients did not maintain the levels of regulatory T-cells that were present before LT.

The results of other studies of mice treated with rapamycin have suggested that antigen-specific T-cells responding to a pathogen express CD62L, which is associated with the development of a memory phenotype, whereas antigen-specific T-cells responding to a graft do not express this marker^[131]. Thus, minimizing the generation of memory cells by treatment with an mTORi could decrease graft rejection responses, and indirectly promote an environment where tolerance could be established.

IDENTIFYING TOLERANT PATIENTS: A BIOMARKER SIGNATURE

A significant number of patients may become opera-

tionally tolerant after LT^[6]; however, identifying tolerant patients before drug withdrawal is the purpose. Thus, researchers have focused on identifying biomarkers of tolerance that would aid the clinician in detecting tolerant individuals and help to elucidate molecular mechanisms of tolerance and provide therapeutic targets^[132] (Table 1).

At the beginning, the studies performed to identify biomarkers of tolerance in LT employed immunophenotyping by flow cytometry and gene expression profiling of blood samples^[40,42,43,133,134]. These studies were made in a retrospective and cross-sectional fashion, where operationally tolerant recipients defined as patients with stable graft function after IS withdrawal were compared to recipients under maintenance IS who suffered a rejection episode during drug weaning process (non-tolerant patients). Mazariegos *et al.*^[134] demonstrated a significant increase in the ratio of peripheral blood monocytoïd dendritic cells (mDC) to plasmacytoid dendritic cell (pDC) precursors in tolerant patients compared to healthy controls and those on maintenance IS. In other reports, tolerant patients exhibited increased numbers of Tregs, and an increase in the $\nu\delta 1/\nu\delta 2$ T-cell subset ratio^[133,135]. One of these groups showed, in their cohort of pediatric liver transplant recipients, that the $\gamma\delta$ T cell signature previously noted in peripheral blood mononuclear cells (PBMC) also characterized intra-graft analysis and also showed significant accumulation of Treg in liver allograft biopsy samples of tolerant *vs* non-tolerant recipients^[133]. These findings were corroborated recently in an adult cohort of tolerant subjects who underwent prospective withdrawal of IS^[136].

While both peripheral blood immune cell phenotyping and cross-platform gene expression profiling showed tolerance to be associated with increases in B-cell-related transcripts, and in some reports, a skewing towards transitional and naïve B-cell repertoires^[137,138], biomarkers associated with liver allograft tolerance are predominantly related to natural killer cells and $\gamma\delta$ T cells in blood, and genes related to iron homeostasis in the graft. Robust highly specific gene signatures have been developed as biomarkers associated with liver allograft tolerance^[139]. The group of Martinez-Llordella *et al.*^[43] was the first to use microarray technology for the gene expression profiling of PBMC from operationally tolerant liver transplant recipients. They compared in a retrospective cross-sectional study gene expression profiles in the peripheral blood of tolerant and non-tolerant liver transplant recipients with healthy controls. They found that clinically tolerant patients could be identified not only by a signature of genes encoding several cell surface receptors expressed by NK, CD8+ and $\gamma\delta$ T cells as well as proteins involved in halting cell proliferation, but also by the expansion of CD4+CD25+Foxp3+ natural regulatory T cells (nTregs) and $\gamma\delta$ TCR+ (especially $\nu\delta 1$ TCR+) T cells in the peripheral blood. This genomic and immunological footprint of operational tolerance was subsequently validated in an independent cohort of 23 additional recipients^[42]. Our group reported one of the first prospective IS withdrawal studies analyzing the expression of FOXP3 in peripheral blood Tregs during withdrawal of IS in liver transplant recipients receiving cyclosporine A^[44]. An increase in the frequency of CD4+CD25high cells was observed when IS was withdrawn in tolerant liver transplant recipients. Any significant difference in this population of cells was not observed in the non-tolerant group. In addition, tolerant patients exhibited an increase in FOXP3 mRNA expression of peripheral blood mononuclear cells before complete IS withdrawal that continued even when IS therapy was stopped.

More recently, Bohne *et al.*^[40] reported the results of the first prospective IS withdrawal trial in liver transplant recipients including blood and liver tissue transcriptional biomarker studies. In that study, 98 liver recipients completed the trial: 57 experienced rejection and 41 were successfully weaned. Sequential blood and/or liver tissue samples from 75 recipients were analyzed with whole-genome microarrays and quantitative polymerase chain reaction. While PBMC gene analysis again corroborated the enrichment of natural killer and $\gamma\delta$ T cell transcripts, additionally, before initiation of drug withdrawal, operationally tolerant and non-tolerant groups differed in the intra-graft expression of genes related to iron metabolism; tolerant patients also had higher serum levels of hepcidin and ferritin as well as increased iron deposition within hepatocytes. More important is the fact that certain hepatic tissue gene expression patterns had a high predictive value of the outcome of IS withdrawal in an independent

set of patients. These results suggest a critical role for iron metabolism in the regulation of human intra-graft alloimmune responses and provide a set of biomarkers to enroll the liver transplant patients into drug weaning trials with higher probability of success^[140].

MicroRNAs (miRNAs) constitute a key regulatory component of immune system development and function. In a recent study, Vitalone *et al.*^[141] found in a rat experimental model of LT an increased expression of miR-142-5p and miR-181a in liver tissue and proposed that these miRNAs represented 2 potential biomarkers associated with tolerance. This study demonstrated the need for ongoing evaluation to delineate the role of individual miRNAs within the context of larger patient cohorts.

Recently, several promising biomarkers have been identified for determining patient alloreactivity and tolerance. A consensus document that aims to help tailor IS has been developed by the Biomarker Working Group of the International Association of Therapeutic Drug Monitoring and Clinical Toxicology^[142].

CLINICAL RELEVANCE OF TOLERANCE IN LIVER TRANSPLANTATION: DOES IMMUNOSUPPRESSION WITHDRAWAL REDUCE THE COMPLICATIONS RESULTING FROM ITS USE?

Regardless of the progress made in recent years in OT, it would be necessary to define in a controlled and prospective way different aspects that arise as questions from the patient's bedside: (1) Is it possible to withdraw IS in patients with LT? (2) Is it dangerous for patients to be subjected to an IS withdrawal protocol? (3) Is IS withdrawal beneficial for patients? and (4) Is there any parameter that during IS withdrawal process allows recognizing the group of patients who can be subjected to IS withdrawal? The first cases of OT after LT were documented by Starzl *et al.*^[143] in the early 1990s. Based on the finding that 11 LT patients had stopped taking IS medication due to lack of treatment adherence or post transplant lymphoproliferative disease, the authors designed a prospective study on intentional withdrawal of IS in LT patients and toxicity associated with IS^[144]. In 18 (19%) of the 95 patients included in the study, IS could be completely withdrawn without causing alterations in liver function up to 2.2 years after inclusion. Since then, various studies have been published in which complete IS withdrawal in LT patients was attempted according to a pre-established protocol. Undoubtedly, intentional IS withdrawal protocols in LT without the use of presumably tolerogenic treatments are the largest in number and were the basis for establishing the proof of the concept of OT. Overall, OT was obtained in 23% of the patients without tolerogenic protocols, all of whom were selected for different reasons;

however, they were generally chosen because of adverse effects of immunosuppressive medication. The strategies investigated up to now, aimed at obtaining an IS-free state, are numerous and heterogeneous in terms of concept, rationale, patient age, underlying LT indications, objectives, type of LT (cadaver or living donor), duration of IS withdrawal period, duration of follow up, presence or absence of donor cell chimerism, tools used to measure tolerance mechanisms, *etc.* Nevertheless, the literature published to date can be summarized by maintaining that a permanent state of OT can be obtained in some patients undergoing LT for non-immunological underlying diseases and that those patients who do not achieve OT and experience rejection are not exposed to a greater risk of graft loss or death.

The first two prospective multicenter monitored clinical trials of IS withdrawal in pediatric and adult patients with LT have been recently published. The study of Benítez *et al.*^[145] is the first prospective multinational study of IS withdrawal in adults. This study included 102 patients out of 500 who were initially analyzed after IS withdrawal for a period between 6 and 9 mo. The primary goal of this study was to define the frequency of operational tolerance and the secondary objectives were based on mortality, graft loss, severity of rejection episodes, time between the start of IS withdrawal and rejection, histological liver changes after IS withdrawal, normalization of graft dysfunction after rejection onset and possible change in the side effects of IS followed by a 36 mo monitoring after inclusion in the study. Its main results and conclusions were:

A 40.2% (41/102) of patients with treatment intention or 41.8% (41/98) of patients by protocol compliance achieved OT, which was stable at least for 49 ± 7.7 mo of follow up.

Not all of the patients analyzed (500) were included in the study. Therefore, the applicability of this IS withdrawal strategy was only 20.4% (102/500).

The non-tolerant patients (57; 58%) were always this way during IS withdrawal and not after finishing withdrawal. Concerning these patients, rejection was mild or moderate in most of them and only severe in 5%. In addition, there were not any cases of chronic rejection and liver dysfunction was resolved in most cases with basal IS restoration or with association of low or moderate doses of steroids.

There were not any changes in comorbidities or in tolerant and non-tolerant patients after a follow-up period of 36 mo.

In tolerant patients, there was an increase of lobular inflammation beginning one year after IS withdrawal, not observed three years after such withdrawal.

One of the most important findings of this study is that the best tolerance predictor after LT is time. It is still more striking to notice that 79.2% of those who had had their graft for 10.6 years or more achieved IS withdrawal, indicating a propensity to develop

tolerance over time. Nevertheless, the probability of tolerance was zero in patients with less than 6 years from LT and who were under the age of 49.

The results of this study demonstrate the real possibility of withdrawing immunosuppressive drugs in a higher proportion of patients with LT than the previously known of 20%, especially the more time has passed after the transplantation. The study has some limitations that the authors themselves acknowledge, as the possible bias because of the strict selection criteria that result in low applicability of this strategy. Furthermore, the lack of clinical benefit in terms of improvement of the side effects of IS requires a closer monitoring to study if it occurs. The findings of this study are consistent with the other large prospective study carried out in 20 pediatric transplant patients with parental living donor^[146]. In this study, 60% of patients reached OT and these patients had been transplanted longer (median, 100.6 mo) than those non-tolerant patients (median, 73 mo). In addition, the study confirms a higher rate of OT in pediatric patients than in adults, as previously demonstrated.

The first two questions posed are clearly answered, both by Benítez *et al.*^[145], and by Feng *et al.*^[147], and it can be asserted that OT is possible and frequent the more time passes from LT and it is not particularly dangerous when done in a controlled way. Nevertheless, a longer follow up is necessary, since sometimes rejection can occur several years after IS withdrawal. However, it is more difficult to answer the question about whether IS withdrawal is beneficial for the patient, since neither study showed benefits.

A major focus of IS reduction or withdrawal has been the long-term effects. Few of the studies performed so far have provided a detailed analysis of the impact of IS withdrawal on pre-existing complications derived from the long-term administration of immunosuppressive drugs and the side effects associated with them (Table 2). In only one study the aim was to evaluate the feasibility of gradual withdrawal of IS in liver transplant recipients and to examine the impact of IS withdrawal on renal function and cardiovascular risk factors^[148]. In this study, IS withdrawal was safely achieved in selected liver transplant patients and improved not only kidney function but also other CyA-associated side effects such as hypercholesterolemia, hyperuricemia, hypertension and diabetes control. However, longer follow-up periods are needed to confirm the benefits of IS withdrawal in liver transplant patients and to observe whether there are problems with chronic rejection after complete withdrawal of immunosuppressive drugs. Only one study has examined the effect of IS withdrawal in hepatitis C virus-positive recipients^[146]. This study showed improvement in fibrosis after withdrawal, similar to that observed with successful post-LT interferon therapy. However, this preliminary study has not been replicated, and a follow-up study almost 3 years later did not show any histological differences.

Table 2 Impact of immunosuppression withdrawal on preexisting complications in liver transplantation

Author (year)	Ref.	No. of patients	Rational for IS withdrawal	Description of impact on preexisting complications	Impact on preexisting complications in tolerant patients
Mazariegos (1997)	[144]	95	Chronic IS-related toxicity	Yes	No changes in renal function or hypertension. Higher survey scores of patients well being
Devlin (1998)	[2]	18	Chronic IS-related toxicity	No	-
Takatsuki (2001)	[161]	63	30 PTLT	No	-
Eason (2005)	[162]	18	Patients who expressed a desire to discontinue IS	No	-
Tryphonopoulos (2005)	[163]	104	Role of DBMI in IS withdrawal in LT	No	-
Orlando (2008)	[146]	34	Impact of IS withdrawal on HCV disease in LT	Yes	Less cardiovascular or infectious diseases
Pons (2009)	[148]	22	Chronic IS-related toxicity	Yes	Renal function, hypertension, hypercholesterolemia, hyperuricemia, hypertension and diabetes control improved No changes in comorbidities
Feng (2012)	[147]	20	Chronic IS-related toxicity	Yes	-
de la Garza (2013)	[158]	22	Chronic IS-related toxicity	No	-
Benitez (2013)	[145]	102	Chronic IS-related toxicity	Yes	No changes in comorbidities

IS: Immunosuppression; PTLT: Post-transplant lymphoproliferative disorder; HCV: Hepatitis C virus; LT: Liver transplantation; DBMI: Donor bone marrow infusion.

In this study, tolerant individuals were euglycemic and more intolerant individuals developed new onset diabetes that required specific treatment. Finally, significantly more intolerant patients were suffering from either cardiovascular or infectious diseases. Yoshitomi *et al.*^[149] found that grafts from operationally tolerant living donor LT recipients exhibited more fibrosis, ductular reactions, and decreased luminal diameter of bile ducts as compared to patients under IS treatment, and that these abnormalities improved after reintroduction of low-dose IS. However, these data should be cautiously interpreted due to the substantial difference in post transplantation time between the two groups.

A limitation of all withdrawal studies is the absence of prospectively followed, IS-maintained patients as control cohorts. Understanding the true clinical benefits of withdrawal rather than comparing long-term outcomes and IS-related effects in tolerant vs intolerant recipients is likely to be more useful when comparing such outcomes of intolerant vs IS-maintained or IS-minimized patients as control cohorts. The potential impact of IS minimization or withdrawal protocols on long-term subclinical histological graft damage (*e.g.*, idiopathic chronic hepatitis and/or progressive fibrosis) also remains to be properly investigated. This is relevant considering that most protocol biopsy studies have revealed substantial histological abnormalities in long-term surviving liver recipients with unremarkable liver function tests.

CONCLUSIONS AND FUTURE CHALLENGES

The future of LT should be focused on the reduction of side effects due to immunosuppressive drugs in order to improve quality of life with preservation of the viability of the liver graft. Tolerance is a reality in a reduced number of patients, so new treatments aimed to increase tolerance of the liver allograft have to be developed. Cell therapy with *ex vivo* expanded Tregs is currently being tested to induce LT tolerance. The effects of mesenchymal stromal cell infusions are also being explored, trying to improve preservation injury, preventing ischemic cholangiopathy, or facilitating IS minimization. While these are very promising studies, key issues related to dosing, timing or most appropriate adjunctive IS will need to be clarified before large scale clinical applications can be considered. Looking into the future, conventional immunosuppressive drugs will likely remain as principal therapy after LT. Some selected patients will not need IS due to induced or spontaneously developed tolerance. In the remaining recipients, IS will be administrated according to the quality of the graft, inflammatory status, or degree of cellular or humoral sensitization.

Defining new biomarkers to assess the individual immune status of a transplant patient to fine tune the immunosuppressive therapy is the key to improve graft and patient survival. Many biomarkers have not yet been validated in comprehensive prospective

clinical trials and the proposed clinical decision limits are frequently based on retrospective and single center experiences. Molecular profiling is evolving at unprecedented rates, as are the bioinformatic techniques required to enable the handling of the vast data pools generated. Definitive substantiation of the clinical utility of any of the discussed biomarkers rests on their successful application in prospective, randomized trials of biomarker-led IS weaning. Lastly, it is becoming evident that a single biomarker cannot be able to reflect all the alterations of the immune system associated with LT. Therefore, a panel of different biomarkers will be needed to properly evaluate the immunological suppression and to modify immunosuppressive treatment according to patient needs.

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Pathophysiology of colorectal peritoneal carcinomatosis: Role of the peritoneum

Lieselotte Lemoine, Paul Sugarbaker, Kurt Van der Speeten

Lieselotte Lemoine, Kurt Van der Speeten, Department of Medicine and Life Sciences, Hasselt University, 3500 Hasselt, Belgium

Lieselotte Lemoine, Kurt Van der Speeten, Department of Surgical Oncology, Ziekenhuis Oost-Limburg, 3600 Genk, Belgium

Paul Sugarbaker, Washington Cancer Institute, Washington Hospital Center, Washington DC 20010, United States

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Correspondence to: Kurt Van der Speeten, MD, PhD, Department of Surgical Oncology, Ziekenhuis Oost-Limburg, Schiepse Bos 6, Genk, Limburg, 3600, Belgium. kurt.vanderspeeten@zol.be
Telephone: +32-89-326524

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Abstract

Colorectal cancer (CRC) is the third most common cancer and the fourth most common cause of cancer-related death worldwide. Besides the lymphatic and haematogenous routes of dissemination, CRC frequently gives rise to transcoelomic spread of tumor cells in the peritoneal cavity, which ultimately leads to peritoneal carcinomatosis (PC). PC is associated with a poor prognosis and bad quality of life for these patients in their terminal stages of disease. A loco-regional treatment modality for PC combining cytoreductive surgery and hyperthermic intraperitoneal peroperative chemotherapy has resulted in promising clinical results. However, this novel approach is associated with significant morbidity and mortality. A comprehensive understanding of the molecular events involved in peritoneal disease spread is paramount in avoiding unnecessary toxicity. The emergence of PC is the result of a molecular crosstalk between cancer cells and host elements, involving several well-defined steps, together known as the peritoneal metastatic cascade. Individual or clumps of tumor cells detach from the primary tumor, gain access to the peritoneal cavity and become susceptible to the regular peritoneal transport. They attach to the distant peritoneum, subsequently invade the subperitoneal space, where angiogenesis sustains proliferation and enables further metastatic growth. These molecular events are not isolated events but rather a continuous and interdependent process. In this manuscript, we review current data regarding the molecular mechanisms underlying the development of

colorectal PC, with a special focus on the peritoneum and the role of the surgeon in peritoneal disease spread.

Key words: Peritoneal carcinomatosis; Pathophysiology; Peritoneal metastatic cascade; Cytoreductive surgery; Peritoneum; Hyperthermic intraperitoneal peroperative chemotherapy

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Core tip: Colorectal peritoneal carcinomatosis (PC) is associated with poor prognosis and bad quality of life for patients in their terminal stages of disease. A loco-regional treatment, combining cytoreductive surgery and hyperthermic intraperitoneal peroperative chemotherapy, is associated with significant morbidity and mortality. Therefore, a comprehensive understanding of the molecular events involved in peritoneal disease spread and subsequent careful patient selection is paramount to avoid unnecessary toxicity. This manuscript highlights current data regarding the molecular mechanisms underlying the development of colorectal PC with a special focus on the peritoneum and the role of the surgeon in peritoneal disease spread.

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INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer and the fourth most common cause of cancer-related death worldwide^[1,2]. Besides the lymphatic and haematogenous routes of dissemination, CRC frequently gives rise to the transcoelomic spread of tumor cells in the peritoneal cavity, which ultimately leads to peritoneal carcinomatosis (PC)^[3]. Colorectal PC is associated with a poor prognosis and bad quality of life for these patients in their terminal stages of disease^[4-6]. Recent genomic profiling studies have demonstrated distinct gene expression patterns, determining CRC spreading to either the liver, the peritoneum or both^[7,8].

The precise incidence of PC is unknown due to the low sensitivity of preoperative imaging techniques (CT, MRI, PET, PET/CT) and heterogeneity of published methods and findings^[9,10]. Using a database of 3019 CRC patients, Jayne *et al*^[11] reported that 8% of these patients presented with synchronous PC and 5% presented with metachronous disease. In a recent population-based cohort study from Stockholm

County in Sweden, 4.3% of 11124 CRC patients were diagnosed with synchronous PC and 4% with metachronous PC^[12]. In the synchronous group, more than 50% of patients presented with peritoneal metastases as the only site of tumor dissemination.

In the past, oncologists and surgeons assumed PC was identical to distant metastases and as such, regarded it as an incurable component of intra-abdominal malignancy only open to palliative treatment options. Systemic chemotherapy in patients with PC resulted in no long-term survival with a median overall survival of 15.2 mo and poor quality of life of these patients in their terminal stages of disease^[13,14]. A new loco-regional treatment modality combining cytoreductive surgery (CRS) and hyperthermic intraperitoneal peroperative chemotherapy (HIPEC) has demonstrated promising clinical results in patients with colorectal PC. In this setting, CRS aims to remove all macroscopic tumor through standardized peritonectomy procedures and multivisceral resections, whereas the subsequent intraoperative chemotherapy seeks to eliminate all residual microscopic tumor^[15]. This novel approach has demonstrated encouraging clinical results in several phase II and III trials^[16-18]. However, CRS and HIPEC are associated with a significant morbidity (grade III-IV complications) of approximately 34% and a 30-d mortality of 4%^[19,20]. Therefore, careful patient selection is paramount to avoid unnecessary toxicity^[21-23]. The aim of this manuscript is to review current data regarding the molecular mechanisms underlying the development of colorectal PC, with a special focus on the peritoneum.

THE PERITONEUM AS THE FIRST LINE OF DEFENCE IN PC

The peritoneum is the largest and most complex serous membrane of the human body^[24]. The visceral peritoneum, covering the intra-abdominal organs and mesenteries, forms a continuous layer with the parietal peritoneum, which lines the abdominal wall and pelvic cavities^[24]. It is composed of a monolayer of mesothelial cells supported by a basement membrane that rests on a layer of connective tissue, also referred to as the submesothelium (Figure 1)^[25].

The mesothelium consists of a monolayer of either flattened, stretched, squamous-like or cuboidal mesothelial cells. The latter can be found in various areas including the liver, the spleen, the "milky spots" of the omentum and the peritoneal side of the diaphragm overlying the lymphatic lacunae (cfr. Attachment to distant peritoneum)^[26-28]. Cuboidal mesothelial cells are also observed within an injured mesothelium. Both squamous and cuboidal mesothelial cells can be distinguished by their ultrastructural differences. Squamous-like mesothelial cells contain few mitochondria, a poorly developed Golgi apparatus and little rough endoplasmic reticulum (RER),

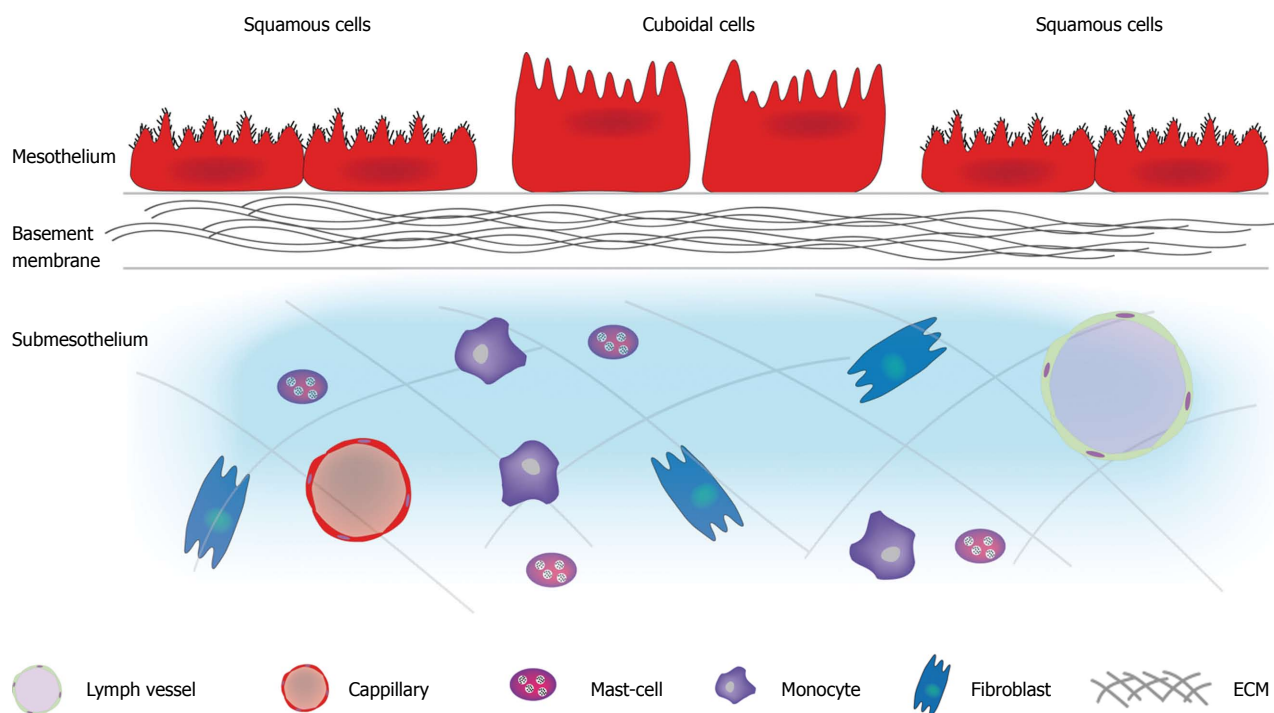


Figure 1 Structure of the peritoneum. The peritoneum is composed of a mesothelium supported by a basement membrane that rests on a layer of submesothelium. The mesothelium consists of a monolayer of either flattened, stretch, squamous-like or cuboidal mesothelial cells. The luminal surface of mesothelial cells has numerous microvilli varying in shape, size and density. Cilia have also been identified on the surface of resting mesothelial cells. The basement membrane consists of a thin laminar network containing type I and IV collagen, proteoglycans and glycoproteins. The submesothelium consists of a complex network of extracellular matrix made up of different types of collagen, glycoproteins, glycosaminoglycans and proteoglycans. Blood vessels, lymphatics, and various cells types (fibroblasts, resident tissue macrophages, and mast cells) are also found in this layer. ECM: Extracellular matrix.

which are located centrally near the round or oval nucleus^[29]. Cuboidal mesothelial cells contain a central prominent nucleolus, abundant mitochondria and RER, a well developed Golgi apparatus, microtubules and microfilaments^[30]. The luminal surface of mesothelial cells has numerous microvilli varying in shape, size and density; increasing the functional mesothelial surface area^[31]. Cilia have also been identified on the surface of resting mesothelial cells^[32]. The mesothelium functions as a dynamic layer that contributes substantially to the structural, functional, and homeostatic properties of the peritoneum^[29]. The underlying basement membrane, a thin laminar network containing type I and IV collagen, proteoglycans and glycoproteins, acts as a selective barrier to macromolecules entering the submesothelial layer^[29]. The submesothelium consists of a complex network of extracellular matrix (ECM) made up of different types of collagen, glycoproteins, glycosaminoglycans and proteoglycans. Blood vessels, lymphatics, and various cell types (fibroblasts, resident tissue macrophages, and mast cells) are also found in this layer^[24,29].

The first major function of the peritoneum is facilitating transport of fluid and cells across the serosal cavities^[33]. The microvilli on the luminal surface of the mesothelial cells play an important role in this process as they increase the surface area and bind fluids in their glycosaminoglycan-rich glycocalyx thereby aiding absorption^[34]. Secondly, the peritoneum

provides a slippery and non-adhesive surface to allow intracoelomic movement^[35]. This slippery and non-adhesive surface is established by the secretion of a small amount of sterile fluid containing phosphatidylcholine produced by each mesothelial cell. Thirdly, the peritoneum acts as a first line of defence in host resistance^[36,37]. The fourth function of the peritoneum involves tissue repair by releasing growth factors^[38,39]. In conclusion, the peritoneum should be considered an organ with a structural and protective function for the contents of the abdominal cavity^[24,25,40].

PATHOPHYSIOLOGY OF COLORECTAL PC

The emergence of PC is the result of a molecular crosstalk between tumor cells and host elements, comprising several well-defined steps. First, individual or clumps of tumor cells detach from the primary tumor and gain access to the peritoneal cavity. In the second step, these free tumor cells become susceptible to the regular peritoneal transport along predictable routes. The third step involves the attachment to the distant peritoneum where the tumor cells, during the fourth step, invade the subperitoneal space. The underlying connective tissue provides the necessary scaffold for tumor proliferation. The final step involves angiogenesis, which sustains tumor proliferation and

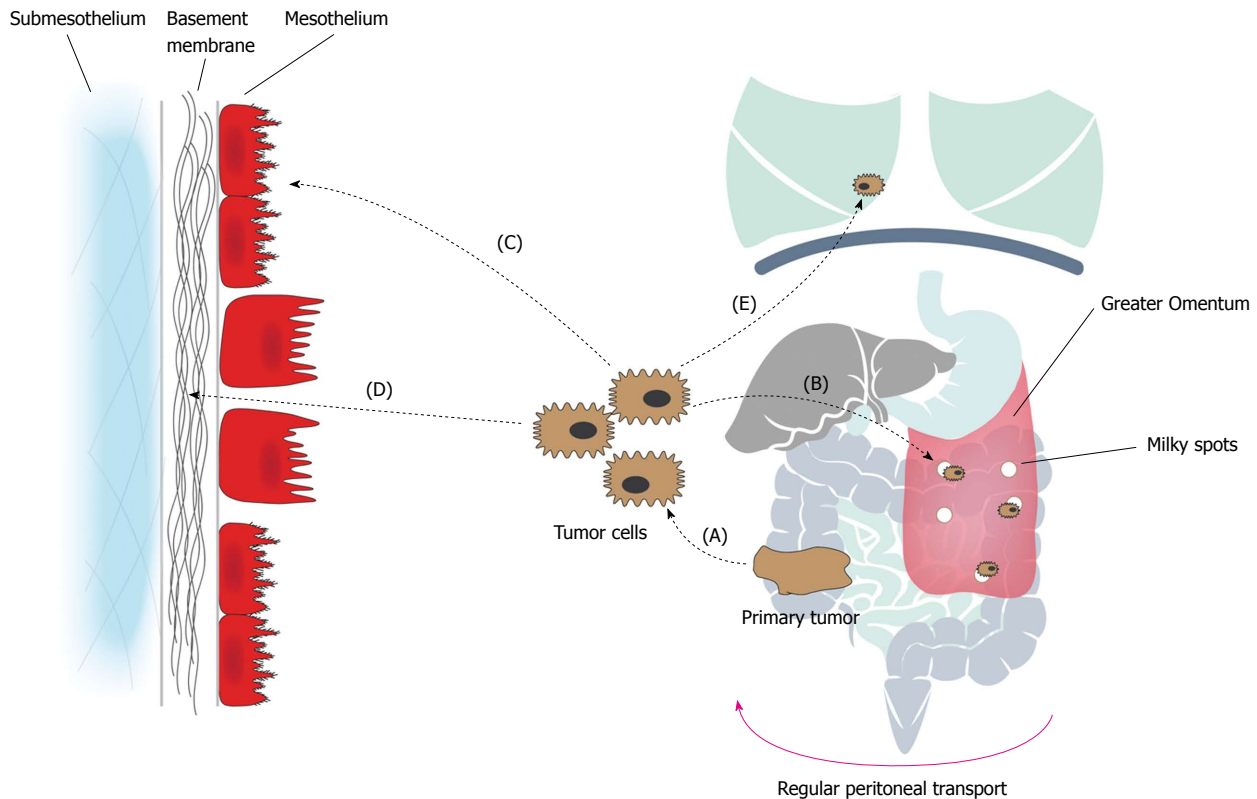


Figure 2 Pathophysiology of colorectal peritoneal carcinomatosis: the peritoneal metastatic cascade. The emergence of PC is the result of a molecular crosstalk between tumor cells and host elements, comprising several well-defined steps. A: Individual or clumps of tumor cells detach from the primary tumor and gain access to the peritoneal cavity. Spontaneous exfoliation of tumor cells from the primary tumor can be promoted by the down-regulation of E-cadherin, increased interstitial fluid pressure, and iatrogenically during surgery; B: The free tumor cells become susceptible to the regular peritoneal transport. Peritoneal transport is due to changes in the intra-abdominal pressure during respiration, gravity and peristalsis of the bowel; which results in a clockwise flow from the pelvis, along the right paracolic gutter and to the subdiaphragmatic space and finally towards the pelvis again; C: Attachment of tumor cells to distant peritoneum occurs via two processes, denominated transmesothelial and translymphatic metastasis. During transmesothelial metastasis, loose tumor cells directly adhere to distant mesothelium through adhesion molecules. During translymphatic metastasis, free tumor cells gain access to the submesothelial lymphatics through lymphatic stomata. Preferential tumor growth in the milky spots of the greater omentum has been observed; D: Tumor cells invade the submesothelium. In areas of absent or rounded (cuboidal) mesothelial cells, tumor cells interact with the laminar network of the basement membrane through integrin-mediated adhesion. Subsequent invasion of the submesothelial tissue occurs via degradation by proteases (MMPs); E: Systemic metastasis. PC: Peritoneal carcinomatosis.

enables further metastatic growth^[41]. It is important to realise that these steps, known as the “peritoneal metastatic cascade”, do not necessarily occur in isolation, but rather describe a continuous and interdependent process (Figure 2, Table 1)^[41].

Detachment of tumor cells from the primary tumor

The metastatic pathway begins with the detachment of individual, or clumps of tumor cells from the primary tumor. This can be the result of spontaneous exfoliation of tumor cells from cancers that have invaded through the full thickness of the bowel wall and its investing serosa^[41,42]. Serosal involvement of colon adenocarcinoma (pT4 stage) is an unfavourable independent prognostic marker for the development of PC^[43-45]. Spontaneous exfoliation of malignant cells can be promoted by the down-regulation of intracellular adhesion molecules on the tumor cell surfaces, more specifically E (epithelial)-cadherin^[46]. E-cadherin belongs to the type I subfamily of cadherins^[47]. The general structure comprises an extracellular part, a membrane-spanning domain and a cytoplasmic

tail. E-cadherin binds homotypically to E-cadherin on neighbouring cells through its Ca^{2+} -dependent extracellular domains. The cytoplasmic tail associates with p120, α -, β -, and γ -catenin, which is responsible for the connection with the actin cytoskeleton and allows in- and outward signal transduction^[46-49]. It has been confirmed that the down-regulation of E-cadherin expression levels is associated with dedifferentiation, progression, and metastasis of CRC^[50,51]. This down-regulated tumor suppressor or metastasis suppressor function has also been reported in gastric^[52,53] and ovarian cancer^[54,55] with PC. Furthermore, reduction of cell-cell adhesion, by the loss of E-cadherin, and the upregulation of mesenchymal N (neural)-cadherin are established hallmarks of the epithelial to mesenchyme transition (EMT)^[56]. This reversible reprogramming process allows cells to separate, lose their apico-basal polarity typical of epithelial cells, demonstrate heightened resistance to apoptosis, and revert to a more motile mesenchymal phenotype^[56]. This is believed to play a major role in the invasion and metastasis of tumor cells^[49,57]. Gargalionis *et al*^[58]

Table 1 General overview molecules/molecular pathways involved in the peritoneal metastatic cascade

Steps peritoneal metastatic cascade	Molecules/molecular pathways
Detachment from the primary tumor	Spontaneous tumor cell shedding: E-cadherin ↓ N-cadherin ↑ EMT PC1 and PC2 ↑ Interstitial fluid pressure ↑ Peroperative seeding tumor cells during surgery
Peritoneal transport	Mucinous ascites Actin microfilament system Lammelipodia, filipodia
Attachment to distant peritoneum	Transmesothelial dissemination: ICAM-1 ↑, PECAM-1, VCAM-1 ↑ TNF- α , IL-1 β , IL-6, IFN- γ β 1 integrin subunit CD43, CD44 Hyaluronan Translymphatic dissemination: Lymphatic stomata Milky spots
Invasion into the subperitoneal space	Rounding of mesothelial cells: HGF/SF ↑ c-Met ↑ Destruction of the mesothelial monolayer: Tumor-induced apoptosis Fas ligand/Fas Adherence to the basement membrane: Integrines Invasion of the peritoneal-blood barrier: MMP-1, MMP-2, MMP-7, MMP-9, MMP-13, MMP-14 ↑ TIMP-1, TIMP-2, TIMP-3, TIMP-4 uPA/uPAR plasminogen activator inhibitor -1 and -2
Proliferation and angiogenesis	Proliferation: EGFR, EGF, TGF α IGF-1, IGF-Binding Protein-3 Angiogenesis: HIF-1 α , HIF-1 β VEGF/VEGFR

E-cadherin: Epithelial-cadherin; N-cadherin: Neural-cadherin; EMT: Epithelial to mesenchyme transition; PC: Polycystin; ICAM: Intercellular adhesion molecule-1; PECAM: Platelet-endothelial cell adhesion molecule-1; VCAM-1: Vascular adhesion molecule-1; TNF- α : Tumor necrosis factor- α ; IL-1 β : Interleukin-1 β ; IL-6: Interleukin-6; IFN- γ : Interferon- γ ; CD43: Sialophorin; HGF: Hepatocyte growth factor; SF: Scatter factor; MMP: Matrix metalloproteinases; TIMP: Tissue inhibitor metalloproteinases; uPA: Urokinase plasminogen activator; uPAR: Urokinase plasminogen activator receptor; EGFR: Epidermal growth factor receptor; EGF: Epidermal growth factor; TGF α : Tumor growth factor α ; IGF-1: Insulin like growth factor-1; HIF: Hypoxia inducible factor; VEGF: Vascular endothelial growth factor; VEGFR: Vascular endothelial growth factor receptor.

report that overexpression of the epithelial polycystins PC1 and PC2 in a human colon carcinoma cell line, HCT116, is able to induce EMT-related alteration in E-cadherin, N-cadherin, Snail and Twist (EMT trigger) mRNA expression. PC2 exogenous expression was also found to increase cell migration^[58]. PC1 and PC2 are both membrane-spanning proteins, which were

first identified as mechanosensors involved in the pathophysiology of polycystic kidney disease^[59]. PC1 is a mechanosensor with G-protein coupled receptor properties that perceives extracellular mechanical signals and translates them into biochemical responses^[60-63]. PC2 constitutes a mechanosensitive Ca²⁺ channel^[64-67]. Both receptors physically interact through their C-terminal, forming heterodimeric complexes at the cell membrane and at primary cilia^[68,69].

Secondly, spontaneous tumor cell shedding from the primary tumor can also occur due to increased interstitial fluid pressure, a well-known phenomenon in solid tumors such as in CRC. Hayashi *et al*^[70] reported that the pressure in the tumor is important, not only for the number of tumor cells shed but, also for the size of emboli shedding into lymphatics around the primary tumor. Interstitial hypertension can be the result of high osmotic pressure, increased vessel permeability and hyperperfusion, rapid cell proliferation, lack of effective lymphatic drainage, hyperplasia around blood vessels and increased production of ECM components^[71].

Thirdly, peroperative seeding of viable tumor cells can also be induced iatrogenically during surgery, when the tumor is inadvertently ruptured, opened or cut into. Alternatively, the presence of tumor cells in the peritoneal cavity can be the result of transected lymphatics and blood vessels during the course of surgical resection^[72].

Using peritoneal lavage cytology, several studies have aimed to assess the presence of free peritoneal tumor cells in gastric and CRC patients. They relate the presence of these cells to various clinical and pathological parameters, including locoregional recurrence rate and survival^[73-77]. A wide range of 2.1% to 52% positive peritoneal cytology is reported across several studies, which reflects the heterogeneity of the techniques and the timing to detect malignant cells in peritoneal washes (Table 2)^[45,76,78]. Lloyd *et al*^[75] and Altomare *et al*^[79] performed both pre and post resection peritoneal lavage cytology analysis using polymerase chain reaction. They both report a similar pre-resection rate of positive cytology, 12% to 14%, but differ in the post resection detection rate, 3%^[79] and 20%^[75]. Recently, Bae *et al*^[76] performed peritoneal lavage cytology in patients with CRC without distant metastasis who underwent curative resection, immediately after making a midline abdominal incision and just before manipulation of the tumor. They reported a rate of positive cytology of 4.1%.

Peritoneal transport of tumor cells

Once the tumor cells have been detached from the primary tumor and seeded in the peritoneal cavity, they become susceptible to the regular peritoneal transport. Previously, it was assumed that intraperitoneal cancer dissemination was a random process independent of the physical and biological properties of the tumor

Table 2 Intra-operative peritoneal lavage: detection method, timing and outcome data

Ref.	Patients, <i>n</i>	Method of detection	Peritoneal lavage fluid	Timing of sampling	Intraperitoneal free cancer cells
Kirstensen <i>et al</i> ^[73]	237	PCR	200-600 mL 0.9% NaCl	After	8.01%
Nishikawa <i>et al</i> ^[74]	410	Cytology	200 mL 0.9% NaCl	Before	7.60%
Lloyd <i>et al</i> ^[75]	125	Immunobead RT-PCR	100 mL 0.9% NaCl	Before	12.80%
				After	29.60%
Bae <i>et al</i> ^[76]	145	Cytology	100 mL 0.9% NaCl	Before	4.10%
Noura <i>et al</i> ^[77]	697	Cytology	100 mL 0.9% NaCl	Before	2.20%
Altomare <i>et al</i> ^[79]	29	Thin-Prep	150 mL 0.9% NaCl	Before	13.80%
				After	2.60%
		RT-PCR	150 mL 0.9% NaCl	Before	37.90%
				After	41.40%
Rossi Del Monte <i>et al</i> ^[78]	48	Cytology	250 mL 0.9% NaCl	Before	0.00%
		Immunofluorescence	250 mL 0.9% NaCl	Before	17.00%
		qRT-PCR	250 mL 0.9% NaCl	Before	42.00%

PCR: Polymerase chain reaction; RT-PCR: Real-time polymerase chain reaction; qRT-PCR: Quantitative real-time polymerase chain reaction.

and the host^[80]. However, several studies report that the direction taken by the loose tumor cells and their ultimate destination are dependent on the anatomic site of the primary tumor and the continued cephalic circulation responsible for the clearance of fluid from the peritoneal cavity^[81-83]. The latter is due to changes in intra-abdominal pressure during respiration, gravity and peristalsis of the bowel; which results in a clockwise flow from the pelvis, along the right paracolic gutter and to the subdiaphragmatic space and finally towards the pelvis again^[40,81]. As a result, certain areas of the peritoneal cavity; the subphrenic region, lesser sac, mesentery, diaphragm and the paracolic gutters, will have an increased risk of occurrence of metastases^[80]. Carmignani *et al*^[81] and Hugen *et al*^[82] report the presence of mucinous ascites to be a prominent facilitator of widespread intraperitoneal cancer distribution with colonic mucinous adenocarcinoma and mucinous colonic adenocarcinoma having different peritoneal surface distribution patterns. The presence of peritoneal adhesions and fibrin entrapment resulting from a surgical trauma are also influencing factors in peritoneal transport. Moreover, during the EMT, malignant tumor cells gain migratory and invasive properties that involve a dramatic reorganisation and activity of the actin microfilament system resulting in the formation of actin-rich membrane protrusions: lamellipodia and filipodia. This process is stimulated by pathological expression of growth factors, their receptors and signalling intermediates, which are the products of proto-oncogenes^[56,84].

Attachment to the distant peritoneum

The ultimate destination of intraperitoneal dissemination depends not only on the physical and biological properties of the free tumor cells but also on the tissue that will harbour the metastatic implantation. The attachment of free CRC cells to the distant peritoneum can occur *via* two processes, denominated transmesothelial and translymphatic metastasis.

During transmesothelial dissemination, loose tumor

cells directly adhere to the distant mesothelium, the innermost layer of the peritoneum. The possible role of adhesion molecules in tumor-mesothelial interactions has been investigated, based on parallels drawn between the mesothelial cell and the endothelial cell^[47]. The mesothelium expresses a distinct pattern of adhesion molecules, which are known to play an important role in leukocyte traffic during peritoneal inflammation and are believed to be exploited by invading tumor cells during the peritoneal metastatic cascade^[29].

Mesothelial cells express adhesion molecules belonging to the Immunoglobulin Superfamily: intercellular adhesion molecule-1 (ICAM-1), platelet-endothelial cell adhesion molecule-1 (PECAM-1) and vascular adhesion molecule-1 (VCAM-1)^[85-87]. Several pro-inflammatory cytokines released following surgery or secreted by circulating tumor cells [tumor necrosis factor- α , interleukin (IL)-1 β , IL-6 and interferon- γ] are known to cause a beneficial environment for the tumor-mesothelial interactions^[88-93]. These cytokines enhance the expression of the adhesion molecules, ICAM-1 and VCAM-1, on mesothelial cells and induce the contraction of mesothelial cells, thereby exposing the basement membrane^[94]. In these areas of absent or rounded mesothelial cells, the interaction between the tumor cells and the laminar network of the basement membrane is mediated through the β 1 integrin subunit^[95-97]. In an *in vitro* study, Ziprin *et al*^[98] demonstrated tumor-mesothelial adhesion by an interaction between mesothelial ICAM-1 and tumor expressed CD43 (sialophorin).

The glycosaminoglycan, hyaluronan, is secreted by the mesothelial cells and subsequently assembled into a pericellular coat^[37]. First, the hyaluronan coat protects the mesothelium from vital infections and the cytotoxic effect of lymphocytes. Secondly, hyaluronan is involved in tumor-mesothelial adhesion through the interaction with tumor expressed CD44^[29,99]. This interaction is an important step in the peritoneal metastatic cascade of ovarian, colon and CRC^[100,101]. CD44 is a cell surface

glycoprotein, which is widely expressed in several non-neoplastic cells as well as neoplastic cells and is involved in migration of cells, homotypic and heterotypic cell-cell adhesion. The *cd44* gene is composed of 20 exons, 10 of which are variably expressed^[102,103]. The smallest and most abundant isoform is the standard form, CD44s. Alternative splicing of 10 variant exons, which account for sequences located in the extracellular part of the CD44, results in the expression of CD44v1 up to CD44v10^[104]. The variant isoforms CD44v3 and CD44v6 are believed to play a role in the metastatic cascade of CRC. Expression of CD44v6 is largely restricted to the advanced stages (T3/T4) of CRC and was higher in metastatic cancer than in nonmetastatic cancer^[102,105]. Saito *et al.*^[106] investigated the clinical importance of CD44s and CD44v6 and their relevance to EMT in 113 patients with stage II/III CRC treated with curative surgery. They report that high expression of CD44v6 is an independent poor prognostic factor for disease-free survival and overall survival.

During translymphatic dissemination, loose tumor cells gain access to the submesothelial lymphatics through openings at the junction of two or more mesothelial cells, the lymphatic stomata. Lymphatic stomata are small openings of lymphatic capillaries, which are involved in immunoregulation but most importantly serve as drainage channels for active absorption of fluids and cells from the serous cavities. They can be found in the greater omentum, appendices epiploicae of the colon, the peritoneal side of the diaphragm, falciform ligament, Douglas pouch and the small bowel mesentery^[107]. Specialized structures, called "milky spots", are also found in these anatomical regions, distributed around the lymphatic stomata. Milky spots are immunocompetent cell aggregates, which resorb peritoneal fluid through their lymphatic stomata and mainly serve as gateways for and providers of macrophages for the abdominal cavity^[108-110]. They play a role in the formation of PC as they provide a highly vascular microenvironment, which permits early survival of circulating tumor cells. The production of VEGF by the mesothelium in the milky spots also promotes angiogenesis, contributing to preferential tumor growth in the milky spots^[111]. However, the precise mechanisms are not well understood. Lopes Cardozo *et al.*^[112] investigated the spread of the syngeneic CC531 colon cancer cells in Wag/Rij rats, after inoculation in the abdominal cavity. They observed tumor cells in the milky spots of the greater omentum within 4 h after intraperitoneal inoculation, demonstrating preferential tumor growth in these immune aggregates.

Invasion into the subperitoneal space

For adhered tumor cells to invade the subperitoneal space, they must first penetrate the mesothelial monolayer. This can occur either at areas of peritoneal discontinuity, by invading the intercellular spaces between adjacent rounded mesothelial cells or by

destroying the monolayer.

Rounding of mesothelial cells occurs in response to several pro-inflammatory cytokines, thereby exposing the basement membrane^[97]. Hepatocyte growth factor/scatter factor (HGF/SF) produced by mesothelial cells induces detachment, motility and proliferation of these cells in the process of mesothelial wound repair^[39]. Binding of HGF to its tyrosine kinase receptor, encoded by the c-MET proto-oncogene, initiates an invasive growth program^[113]. This program is required during embryonic development for tissue and organ morphogenesis, but is exploited by tumor cells to promote invasive and metastatic ability^[114,115]. Sawada *et al.*^[116] already reported that overexpression of c-Met is a prognostic factor in ovarian cancer and targeting this receptor in cultured ovarian carcinoma cells inhibited peritoneal dissemination through an $\alpha 5 \beta 1$ integrin-dependent mechanism. In CRC, Osada *et al.*^[117] investigated the effect of HGF on progression of liver metastasis. They reported that malignancy with high c-Met expression and under high level of HGF, led to unfavourable patient prognosis and poor survival. The presence of malignant ascites was also reported to contain factors, which induce changes in the morphology of the mesothelial cells, resulting in the separation of cell-cell contacts and the subsequent establishment of the characteristic round morphology^[94,118].

Destruction of the mesothelial monolayer can occur through tumor-induced apoptosis. Using a three-dimensional *in vitro* model of the human peritoneum, Jayne *et al.*^[94] demonstrated that CRC cell lines rapidly adhered to the outer mesothelial monolayer. The majority of the adhered tumor cells displayed proliferative growth on the mesothelial surface without invasion. A proportion of the tumor cells invaded the mesothelium, which was characterized by apoptosis of the mesothelial cells involving membrane blebbing, cell shrinkage and nuclear fragmentation. Heath *et al.*^[119] explored the role of the death ligand/receptor system, Fas Ligand/Fas, in the process of apoptosis using human mesothelial cells co-cultured *in vitro* with the SW480 colonic cancer cell line. They demonstrated that invasion of the peritoneal mesothelium occurs *via* tumor-induced mesothelial apoptosis, at least in part mediated by a Fas-dependent mechanism.

After penetrating the mesothelium, the tumor cells adhere to the basement membrane through integrin-mediated adhesion. Integrins are calcium/magnesium-dependent heterodimer molecules, consisting of an α and a β subunit, located on the cell membrane. They are involved in both homotypic cell-cell and heterotypic cell-ECM adhesion and mediate in- and outward signal transduction to the actin cytoskeleton *via* cytoplasmic proteins^[47].

Subsequent invasion of the peritoneal-blood barrier, the submesothelial tissue between the peritoneal mesothelium and the submesothelial arterial blood

Table 3 Overview matrix metalloproteinases^[124]

MMP	Name	Producing Cells	Substrates
MMP-1	Collagenase	Fibroblasts, synovial cells, chondrocyte	Collagen I , II , III , X
MMP-2	Gelatinase	Fibroblasts, chondrocyte, mesangium endothelial cells, cancer cells	Gelatin, collagen IV , V , VII , XI Laminin, fibronectin, elastin
MMP-3	Stromelysin-1	Synovial cells, chondrocyte, fibroblasts	Proteoglycan, collagen III , IV , VII , IX , elastin
MMP-7	Matrilysin	Cancer cells, macrophage	Proteoglycan, gelatin, fibronectin elastin, collagen IV , laminin
MMP-8	Neutrophil, Collagenase	Neutrophil	Collagen I , II , III
MMP-9	Gelatinase B	Neutrophil, macrophage, thromboplast osteoclast, cancer cells, T-lymphocyte	Gelatin, collagen III , IV , V $\alpha 2$ chain, Elastin
MMP-10	Stromelysin-2	Cancer cells, T-lymphocyte	Collagen III , IV , V , fibronectin, gelatin
MMP-11	Stromelysin-3	Cancer cells, macrophage, mesangium	Fibronectin, laminin, proteoglycan, gelatin
MMP-12	Metalloestelase	Macrophage	Elastin
MMP-13	Collagenase-3	Chondrocyte, cancer cells	Collagen I , II , III
MMP-14	MT1-MMP	Cancer cells, fibroblasts	Collagen I , II , III , gelatin, Laminin Fibronectin, vitronectin, proteoglycan
MMP-15	MT2-MMP	Cancer cells, fibroblasts	Fibronectin, aggrecan, tenascin
MMP-16	MT3-MMP	Neuronal cell	Collagen III , gelatin, fibronectin
MMP-17	MT4-MMP	Unknown	Unknown
MMP-20	Enamelysin	Odontoblast	Amelogenin, gelatin
MMP-24	MT5-MMP	Unknown	Unknown
MMP-25	MT6-MMP	Unknown	Unknown
TIMP-1		Tissue, extracellular fluid	Complex formation with pro-MMP-9 and MMPs
TIMP-2		Tissue, extracellular fluid	Complex formation with pro-MMP-9, inhibition of MMP-2 degradation

MMP: Matrix metalloproteinases; TIMP: Tissue inhibitor metalloproteinases.

capillaries, occurs *via* degradation by proteases^[120]. Tumor cells, mesothelial cells, surrounding fibroblasts, inflammatory cells and macrophages secrete matrix metalloproteinases (MMPs), which are responsible for the degradation of several ECM components^[121]. Destruction of the peritoneal-blood barrier by these enzymes results from a disturbed equilibrium between the activation of pro- MMPs and their inhibition by tissue inhibitor metalloproteinases (TIMPs)^[47]. In CRC, increased levels of MMP-1, MMP-2, MMP-7, MMP-9, MMP-13 and MMP-14 have been reported to play a role in the formation of PC. The MMPs are a family of zinc- and calcium dependent multifunctional enzymes currently comprising 23 members in humans, either membrane-anchored or secreted^[122,123]. Many MMPs have overlapping substrate specificity and are involved in a network of mutual activation by MMPs and plasmin activation (Table 3)^[124]. Four types of TIMPs (TIMP-1 - TIMP-4) have been reported, which control the activity of the MMPs^[125-129].

Elevated expression of MMP1 has been reported in several studies to be correlated with metastasis, reduced overall and/or disease-free survival^[129-132]. However, some controversy exists regarding the role of MMP-1. Hettiaratchi *et al*^[133] published a study, including 503 CRC patients and 471 healthy individuals, demonstrating that a single nucleotide polymorphism in the *mmp-1* gene promoter resulted in a significantly improved 5-year survival rate.

Groblewska *et al*^[134] suggested that MMP-2 and TIMP-2 play a role in the process of CRC invasion and metastasis. They conducted a study with 72

CRC patients and 68 healthy individuals to assess the serum levels and tissue expression of MMP-2 and TIMP2. MMP-9 has been implicated in the progression, invasion and metastasis of CRC^[135,136]. In a study conducted by Alkhamesi *et al*^[137], bidirectional signalling was reported between mesothelial cells and tumor cells in generating cancer invasion. They demonstrated that the interaction of ICAM with its ligand, CD43, plays a vital role in both peritoneal adhesion of tumor cells and the preparation of the right environment for subsequent invasion by increasing the production of MMPs (MMP-2 and MMP-9).

MMP-7 is the smallest member of the MMP family and has been proposed to fulfil a dual role in the progression of peritoneal metastases. On the one hand, MMP-7 can have a potential role in tumor invasion and metastasis by degrading basement membrane and submesothelial components. On the other hand, MMP-7 can promote the development and progression of tumor cells by inhibiting tumor cell apoptosis, decreasing cell adhesion and inducing angiogenesis^[138].

Yamada *et al*^[139] reported MMP-13 as a useful predictor of liver metastasis in patients diagnosed with CRC.

Another mediator in the degradation of peritoneal-blood barrier is the urokinase plasminogen activating system, consisting of the urokinase plasminogen activator receptor (uPAR) and the urokinase plasminogen activator (uPA). uPA is a serine protease, which upon activation of the pro-enzyme (pro-uPA) catalyses the reaction in which plasminogen is converted to

plasmin. Plasmin is in turn responsible for the degradation of several ECM components and the activation of pro-MMPs^[140,141]. The catalytic activity of uPA is controlled by its inhibitors, plasminogen activator inhibitor-1 and plasminogen activator inhibitor-2, through the formation of an enzymatically inactive, trimeric receptor-protease-inhibitor complex^[142]. Seetoo *et al.*^[143] investigated the expression levels of PAS in a series of human CRC tissues and correlated these results with patient outcome. They report uPA and uPAR to be possible independent predictors of liver metastasis, patient overall survival and cancer-specific survival after resection of colorectal tumors.

Proliferation and angiogenesis

A known hallmark of malignant tumor cells is their ability to trigger proliferation. Sustained proliferation is achieved through the production of growth factors and their receptors by tumor cells and their associated stromal cells, inducing autocrine and paracrine loops^[144]. Both the epidermal growth factor receptor (EGFR) and the insulin like growth factor-1 (IGF-1) have been reported to be involved in this process^[7,145].

EGFR belongs to the ErbB cell surface receptor family and can be activated by several ligands including EGF and TGF α ^[146]. Binding of its ligand results in homo- or heterodimerization of various ErbB family members, followed by internalisation of the EGFR receptor complex. Upon autophosphorylation of the EGRF tyrosine kinase domains in the cytoplasmic tails, a transduction signalling cascade is initiated, which in turn regulates tumor cell proliferation, differentiation and survival^[147,148]. Yonemura *et al.*^[149] reported that gastric tumors with synchronous expression of EGF and EGFR had the highest malignant potential, causing autocrine secretions for self-replication. Ziober *et al.*^[150] conducted an *in vitro* assay with CRC cell lines and demonstrated that TGF α activated autocrine circuits with its receptor, EGFR, and determined growth factor independence of CRC cells^[150,151]. Tampellini *et al.*^[152] reported that co-expression of immunoreactive EGFR and TGF α was significantly higher in CRC with distant metastases at diagnosis than in CRC presenting at a lower tumor stage.

IGF-1 and its transmembrane receptor are part of a family of cellular modulators that are important in the regulation of growth and development^[153]. Varghese *et al.*^[7] performed microarray analyses on tumors from patients with CRC metastasized to either the liver or the peritoneum. *IGF-1* was exclusively upregulated in tumor samples of patients with peritoneal metastases. Further evidence of the involvement of IGF-1 was provided by Fuchs *et al.*^[154]. They reported that increased plasma levels of IGF-Binding Protein-3, an endogenous antagonist of IGF-1, were associated with improved treatment response to first-line chemotherapy and a prolonged time to cancer progression.

Angiogenesis, the growth of new blood vessels from pre-existing vessels, is paramount for tumor growth and the formation of metastases. For their survival, tumor cells rely on the delivery of oxygen from pre-existing blood vessels and nutrients by the recruitment of stromal cells. However, when these tumor cells are located more than 150 μ m from the submesothelial capillaries, oxygen and nutrients will not be able to pass the peritoneal-blood barrier resulting in hypoxia-induced apoptosis^[155]. Therefore, angiogenesis is induced through the production of angiogenic factors by tumor cells^[156,157]. Key players in this process are hypoxia inducible factor-1 (HIF-1) and VEGF.

HIF-1 is a heterodimeric protein composed of HIF-1 α and HIF-1 β , which activates the transcription of genes involved in the induction of angiogenesis, including VEGF^[158,159]. HIF-1 β is constitutively expressed and does therefore not depend on the hypoxic status of the cells. The expression of HIF-1 α increases exponentially as oxygen levels decline in the cell^[160]. In the same study discussed above, Varghese *et al.*^[7] reported that *HIF-1 α* was upregulated only in tumor samples from peritoneal metastases. Greijer *et al.*^[161] investigated the expression of HIF-1 in normal colorectal mucosa, adenomas and carcinomas. They observed upregulation of HIF-1 α in colorectal adenomas and carcinomas. Using immunohistochemistry, Wu *et al.*^[162] investigated the clinicopathologic significance of HIF-1 expression in primary human colon cancer. High expression levels correlated positively with an advanced TNM stage and were associated with increased metastatic potential^[162].

The VEGF family constitutes five structurally related proteins, VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor. VEGF-C and VEGF-D are important in the process of lymphangiogenesis, while VEGF-A, VEGF-B and placental growth factor are important in neovascularization^[163-166]. The most potent pro-angiogenic growth factor, VEGF-A binds to its receptors VEGFR-1 and VEGFR-2 and thereby increases endothelial cell survival, proliferation, migration and differentiation^[167,168]. VEGF-A displays sensitivity to hypoxia and its expression in growing tissue is regulated by HIF^[169]. In a study conducted by Shaheen *et al.*^[170], nude mice were injected with KM12L4 human colon cancer cells to generate PC. The simultaneous blockage of the VEGF and the EGF receptors with anti-VEGFR (DC101) and anti-EGFR (C225) resulted in decreased tumor vascularity, growth, proliferation, formation of ascites and increased apoptosis of both tumor cells and endothelial cells. Using immunohistochemistry, Logan-Collins *et al.*^[171] investigated tumor samples from patients undergoing CRS and intraperitoneal hyperthermic perfusion for mucinous adenocarcinoma of appendiceal or colonic origin. They reported that overall survival was better in patients with low VEGF expression than in patients with high VEGF expression.

ROLE OF THE SURGEON IN PERITONEAL DISEASE SPREAD

The above-mentioned pathophysiology describes the formation of PC as a continuous and interdependent process, known as the peritoneal metastatic cascade. The surgeon plays a dual role in this process, as promotor of PC by breaching the mesothelium during surgery but at the same time as opponent of PC by performing CRS and HIPEC.

The role of the surgeon as promotor of PC was first described by Sugarbaker^[22] in what they called the "tumor cell entrapment" hypothesis. This hypothesis explains the rapid peritoneal disease progression in patients who have undergone surgery as sole treatment. In this setting, the surgeon is responsible for: (1) peroperative seeding of malignant tumor cells originating from transected lymphatics and blood vessels; and/or (2) the dissemination of malignant cells when the tumor is inadvertently ruptured, opened or cut into^[25,72]; (3) The peritonectomized surfaces and areas where the peritoneal barrier is disrupted during the course of surgical dissection, provide a favourable niche for the re-implantation of these free tumor cells^[96]. The tumor cells become entrapped in the local fibrin deposition present on the traumatised peritoneal surfaces, where they can progress in the presence of growth factors involved in wound healing^[172,173]. The fibrin is infiltrated by platelets, neutrophils and monocytes as part of this wound healing process. Using a rat model, van den Tol *et al.*^[174] reported that growth of intraperitoneally administered rat colon carcinoma cells was enhanced when injected together with lavage fluid from intra-abdominally traumatized animals. Lee *et al.*^[175] investigated whether the wound healing response after an abdominal incision leads to locally increased MMP-9 activity, thereby contributing to peritoneal metastasis. Using a murine model, they concluded that wound-associated inflammation enhances pro-MMP-9 expression. This in turn plays a key role in the growth and progression of tumor cells associated with peritoneal metastases^[176].

Port-site recurrences or recurrences situated at extraction site skin incisions have been established concerns since the introduction of diagnostic or cancer laparoscopy^[177,178]. First, port-site recurrences are abdominal wall recurrences that occur in the subcutaneous tissue as result of slipping of trocars as well as poor extraction techniques^[179,180]. Second, the increase in intraperitoneal pressure during laparoscopy promotes tumor invasiveness and tumor growth *via* a protease-determined pathway. Using an *in vitro* experiment, Paraskeva *et al.*^[181] reported that the exposure of a human colon carcinoma cell line to a laparoscopic environment enhances the production of MMP-2, MMP-9 and uPA.

The surgeon's role as preventer of PC involves the administration of intraperitoneal chemotherapy following

CRS during the peroperative period. Today's treatment of colorectal PC involves the combined treatment modality of CRS and HIPEC. The rationale behind the use of HIPEC after CRS is to treat viable circulating tumor cells, residual microscopic lesions, and to eliminate viable platelets, leukocytes and monocytes from the peritoneal cavity. The latter reduces the promotion of tumor growth at traumatized peritoneal surfaces during the wound healing process. Combining CRS and HIPEC for the treatment of colorectal PC has demonstrated encouraging clinical results in several phase II en III trials^[16-18]. In a multicentre French trial, Elias *et al.*^[182] report an overall 1-year, 3-year and 5-year survival rate of 81%, 41% and 27%.

CONCLUSION

PC is the result of a complex molecular crosstalk between cancer cells and host elements, comprising several well-defined steps, known as the peritoneal metastatic cascade. Individual or clumps of tumor cells detach from the primary tumor, gain access to the peritoneal cavity and become susceptible to the regular peritoneal transport. They attach to distant peritoneum, invade the subperitoneal space, where angiogenesis sustains proliferation and enables further metastatic growth. It is important to realise that these molecular events do not necessarily occur in isolation, but rather describe a continuous and interdependent process. Current treatment combines CRS and HIPEC. This approach is associated with significant morbidity and mortality. A comprehensive understanding of the molecular events involved in peritoneal disease spread, subsequent careful patient selection and knowledgeable management of peritoneal surface metastases is therefore paramount to avoid unnecessary toxicity.

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Management of patients after recovering from acute severe biliary pancreatitis

Georgia Dedemadi, Manolis Nikolopoulos, Ioannis Kalaitzopoulos, George Sgourakis

Georgia Dedemadi, Manolis Nikolopoulos, Ioannis Kalaitzopoulos, Department of Surgery, Sismanoglio-Amalia Fleming Hospital, Melissia, 15127 Athens, Greece

George Sgourakis, Department of Surgery, Furness General Hospital, Dalton Lane, Barrow-in-Furness, Cumbria LA14 4LF, United Kingdom

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Correspondence to: Georgia Dedemadi, MD, PhD, FACS, Department of Surgery, Sismanoglio-Amalia Fleming Hospital, 14, 25th Martiou str, Melissia, 15127 Athens, Greece. gdedemadi@gmail.com
Telephone: +30-210-6033361
Fax: +30-210-6033361

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Abstract

Cholelithiasis is the most common cause of acute pancreatitis, accounting 35%-60% of cases. Around 15%-20% of patients suffer a severe attack with high morbidity and mortality rates. As far as treatment is concerned, the optimum method of late management of patients with severe acute biliary pancreatitis is still contentious and the main question is over the correct timing of every intervention. Patients after recovering from an acute episode of severe biliary pancreatitis can be offered alternative options in their management, including cholecystectomy, endoscopic retrograde cholangiopancreatography (ERCP) and sphincterotomy, or no definitive treatment. Delaying cholecystectomy until after resolution of the inflammatory process, usually not earlier than 6 wk after onset of acute pancreatitis, seems to be a safe policy. ERCP and sphincterotomy on index admission prevent recurrent episodes of pancreatitis until cholecystectomy is performed, but if used for definitive treatment, they can be a valuable tool for patients unfit for surgery. Some patients who survive severe biliary pancreatitis may develop pseudocysts or walled-off necrosis. Management of pseudocysts with minimally invasive techniques, if not therapeutic, can be used as a bridge to definitive operative treatment, which includes delayed cholecystectomy and concurrent pseudocyst drainage in some patients. A management algorithm has been developed for patients surviving severe biliary pancreatitis according to the currently published data in the literature.

Key words: Biliary pancreatitis; Cholecystectomy; Endoscopic retrograde cholangiopancreatography; Sphincterotomy; Pseudocyst; Walled-off necrosis

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Core tip: There is a paucity of data as to which of the following treatment options, including cholecystectomy, endoscopic retrograde cholangiopancreatography and sphincterotomy, drainage techniques for fluid collections and pseudocysts, or no definitive treatment, is the optimal for patients after recovering from an acute episode of severe biliary pancreatitis. The complexity of pancreatitis regarding its course, patient's performance status, and the variety of available interventions should be taken into consideration, raising the need for multidisciplinary management and individualization of every case.

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INTRODUCTION

The most common cause of acute pancreatitis in many western and Asian countries is cholelithiasis, accounting for 35%-60% of cases^[1]. Most patients experience a relatively benign course of pancreatitis, but 15%-20% of patients suffer a severe attack^[2], which is associated with high morbidity and an estimated mortality rate of 20%-30%^[1]. Severe acute pancreatitis is defined by the presence of organ failure persisting beyond 48 h^[2,3]. As far as the treatment is concerned, the optimum method for late management of patients with severe acute biliary pancreatitis is still contentious and the main question is the correct timing of every intervention. Long-term management of symptomatic cholelithiasis aims at minimizing the risk of new biliary events. In the study of Melman *et al*^[4], > 50% of patients with biliary pancreatitis required laparoscopic or open cholecystectomy as part of the overall management. The American College of Gastroenterology and the International Association of Pancreatology/American Pancreatic Association (IAP/APA) Working Group recommend that definitive treatment in acute biliary pancreatitis should include cholecystectomy as the treatment of choice, thus avoiding recurrent biliary events^[5,6]. However, 25%-50% of patients do not undergo cholecystectomy for a variety of reasons, regardless of current guidelines^[7-9]. In a recent retrospective study including 5079 patients initially treated with sphincterotomy, interval cholecystectomy was the most efficient method for preventing episodes of recurrent biliary pancreatitis and offered the best long-term outcomes^[10]. The timing of cholecystectomy in patients with peripancreatic fluid collections has not yet been determined. Early cholecystectomy raises

the risk of a second general anesthetic or a risk of a second interventional procedure to manage persistent fluid collections. Our searches of the literature have revealed the fact that peripancreatic fluid collection and pseudocysts must be considered in the timing of cholecystectomy after an episode of moderate to severe biliary pancreatitis has not been addressed extensively. Certainly, there are ample reports in the literature regarding the timing of intervention for pseudocyst^[11,12].

Currently available therapeutic options for patients who have survived severe biliary pancreatitis are: (1) conservative management; (2) index cholecystectomy; (3) endoscopic retrograde cholangiopancreatography (ERCP) and sphincterotomy; (4) interval cholecystectomy; (5) intervention for pseudocyst and walled-off necrosis; and (6) a combination of all or some of the above options. We assume that selective use of ERCP and sphincterotomy combined with interval cholecystectomy and concurrent pseudocyst management, if required, is the best option for treating patients after recovering from an acute episode of severe biliary pancreatitis.

LITERATURE SEARCH

A literature search was performed concerning the management of patients after recovering from an acute episode of severe biliary pancreatitis. The electronic databases MEDLINE, PubMed, EMBASE, Cochrane Library and Google Scholar were used to search for relevant articles published in 1976-2016, using the following terms and/or combinations in their titles, abstracts, or keyword lists: acute pancreatitis, biliary pancreatitis, severe acute pancreatitis, pancreatic pseudocysts, index cholecystectomy, interval cholecystectomy, percutaneous pseudocyst drainage, endoscopic pseudocyst drainage, surgical pseudocyst management. The above-mentioned terms were used in [MeSH] (PubMed and Cochrane Library), where applicable; otherwise, they were combined using "AND/OR" and asterisks.

The following exclusion criteria were initially applied to all articles identified: publication of abstract only, case reports, and mean or median follow-up of 6 mo. Inclusion criteria were: observational cohort studies, randomized trials, reviews, meta-analyses, systematic reviews and Cochrane Database Systematic Reviews, studies available in full text, and published in the English language. Further references from the selected articles were reviewed manually to supplement the electronic search for additional relevant articles. The following variables concerning studies that address the management of patients with acute severe biliary pancreatitis were recorded: authors, journal and year of publication, country of origin, trial duration and participant demographics. Data concerning follow-up evaluation, ratios and percentages of morbidity,

mortality, biliary events, recurrent pancreatitis, sepsis and other complications according to each treatment option were recorded in a database (Microsoft Excel spreadsheet).

BACKGROUND DATA

Patients after recovering from an acute episode of severe biliary pancreatitis can be offered alternative options in their management, including cholecystectomy, ERCP and sphincterotomy, or no definitive treatment. Identifying these patients is a major concern because it affects the type and timing of intervention^[13]. The implementation of appropriate treatment is directly correlated with the patients' performance status, as frail elderly patients or patients with an impaired general condition and severe comorbid disease are usually unfit for surgery^[5,6]. One also has to take into account that a number of patients who have survived severe biliary pancreatitis may develop pseudocysts or walled-off necrosis. Patients are generally considered fit for surgery according to their physiological fitness and functional capacity to cope with the above-mentioned procedures/interventions. There is a wide variety of prediction models referred to in the literature and used in different centers^[14,15]. Adequate treatment must be provided according to patients' general condition, offering alternative options, such as percutaneous, endoscopic or surgical treatment^[4,16,17].

Patients unfit for surgery

Conservatively treated patients after recovering from an acute episode of biliary pancreatitis have a significant risk of developing recurrent biliary events^[7,10,18]. A large observational study by El-Dhuwaib *et al*^[9], including 5454 patients, found that patients who did not undergo definitive treatment at index admission had an increased risk of recurrent biliary pancreatitis with a cumulative readmission rate of 4% within 2 wk after discharge, 7.7% within 6 wk and 12.8% within 52 wk.

Patients fit for surgery

Cholecystectomy: Cholecystectomy is the definitive treatment for acute biliary pancreatitis^[5,6]. A significantly decreased risk of recurrent biliary pancreatitis, ranging between 1% and 5.1% is observed in patients who undergo index cholecystectomy, as it does not entirely prevent recurrent disease^[8,9,19]. A population-based study questions the effectiveness of cholecystectomy for preventing recurrent episodes of acute pancreatitis in patients with neither a significant elevation of liver tests on day 1 of acute pancreatitis nor stones or sludge in the gallbladder on initial ultrasound evaluation, as recurrence rates were 34% and 61%, respectively. Recurrent attacks of acute pancreatitis after cholecystectomy were low when acute pancreatitis was associated with significantly elevated liver enzymes on day 1 of index admission^[20].

Open cholecystectomy has a limited role; it can be performed along with debridement of necrotizing pancreatitis, and, in cases where a pancreatic pseudocyst is present, after unsuccessful percutaneous or endoscopic approaches, and in failed laparoscopy^[13,21,22]. Laparoscopic cholecystectomy is the preferred approach, offering all the well-known advantages of minimally invasive procedures. The timing of laparoscopic cholecystectomy after an episode of mild acute biliary pancreatitis is controversial, whereas in severe pancreatitis, current guidelines recommend delaying cholecystectomy until after resolution of inflammatory process, usually not earlier than 6 wk after onset of acute pancreatitis^[5,6,23].

According to the IAP/APA Working Group, in patients with peripancreatic collections, cholecystectomy should be delayed until collections either resolve, or, if they persist > 6 wk, cholecystectomy can be performed safely at this time^[6]. Nealon *et al*^[24] suggest that nonoperative management of the pseudocyst, as a bridge to definitive operative treatment, which includes delayed cholecystectomy and concurrent pseudocyst drainage, can be appropriate for a certain number of patients.

Need for ERCP and sphincterotomy: The AGA Institute Technical Review on Acute Pancreatitis maintains that ERCP should be performed in patients with a high suspicion of a persistent common bile duct stone^[25], and the IAP/APA Working Group claims that ERCP is perhaps advisable in biliary pancreatitis with common bile duct obstruction, and possibly not indicated in predicted severe biliary pancreatitis without cholangitis^[6]. Furthermore, the American College of Gastroenterology guidelines state that ERCP is not required in most patients with biliary pancreatitis who do not present with laboratory or clinical evidence of persistent biliary obstruction^[5]. The majority of patients with biliary pancreatitis have spontaneous passage of stones into the duodenum^[26], possibly rendering ERCP redundant, thus reducing potential associated complications. ERCP-related complications range from 5% to 10% and mortality from 0.2% to 0.5%, therefore, accurate prediction of common bile duct stones is required to avoid unnecessary interventions^[27,28]. The decision to perform ERCP is often taken without substantial supporting evidence and is commonly based on biochemical (presence of cholestatic liver biochemistry) and radiological (dilated common bile duct) criteria, although they are proven to be unreliable factors for detecting the presence of common bile duct stones^[29]. Subsequent to an episode of biliary pancreatitis, ERCP and sphincterotomy can be performed on an elective basis for extraction of impacted biliary stone^[30], but there is no evidence regarding the optimal timing of ERCP in patients with biliary pancreatitis without cholangitis^[6].

Recent noninvasive imaging modalities, such as

endoscopic ultrasound (EUS) and magnetic resonance cholangiopancreatography (MRCP) are at present accurate in detecting intraductal stones and identifying patients with persistent obstruction^[31,32]. Sensitivity and specificity for detecting common bile duct stones for MRCP were 92% and 97%, respectively, and for EUS, 89% and 94%, respectively^[31,32]. The sensitivity of MRCP decreases to about 65% when diagnosing stones < 5 mm, while the sensitivity of EUS does not vary with stone size^[32]. This is particularly important because small stones are a common cause of biliary pancreatitis. EUS is more accurate than MRCP in the detection of intraductal stones < 5 mm, but MRCP is a less invasive method, less dependent on the operator and generally available, so there is no clear predominance of MRCP or EUS^[6]. These imaging modalities are of most importance in deciding which patients can benefit from ERCP and sphincterotomy^[29]. However, no specific evidence exists in the setting of severe acute biliary pancreatitis^[33]. MRCP or EUS instead of ERCP should be carried out for suspected common bile duct stones in patients with biliary pancreatitis in the absence of cholangitis or biliary obstruction^[5,29]. EUS is preferred over MRCP because EUS and ERCP can be performed in a single session if required. Savides noted that, even if MRCP reveals an intraductal stone, it is still worth considering EUS immediately before ERCP^[34] because about 21% of intraductal stones (especially those < 8 mm) can pass spontaneously, which can occur in the interval between MRCP and ERCP^[26]. In a recent review, Anderloni *et al.*^[35] state that EUS has recently been proposed as the new gold standard in the diagnosis of choledocholithiasis, as it is well known that small stones occasionally cannot be detected during ERCP; stones < 4 mm mainly in dilated common bile ducts can be hidden by contrast injection. The use of EUS before ERCP for stones < 4 mm is supported by sufficient evidence, as about two-thirds of ERCPs can be avoided^[36].

Patients with pseudocysts and walled-off necrosis:

Patients with persistent organ failure (> 48 h) in the late phase of severe acute pancreatitis are more likely to develop local complications, while some patients may recover without complications^[3]. According to the revised Atlanta classification, local complications include: acute peripancreatic fluid collection, pancreatic pseudocyst, acute necrotic collection, and walled-off necrosis^[2]. Localized collections persisting for > 4 wk can evolve into either a pseudocyst, if containing fluid only, or walled-off necrosis, if containing solid necrotic material; both within a well-defined wall^[2]. A pseudocyst occurs after the onset of interstitial edematous pancreatitis but also in the setting of acute necrotizing pancreatitis as a result of disconnected duct syndrome, while walled-off necrosis occurs after the onset of necrotizing pancreatitis^[37]. The distinction between these two entities was initiated in the 2012 revised Atlanta classification; therefore, the previously published papers do not contain this differentiation.

Acute peripancreatic fluid collections and pseudocysts are the most frequent complications following acute severe pancreatitis. Pancreatic pseudocysts have been traditionally treated surgically and this approach is still frequently used. The recent trend in the management of symptomatic pseudocysts has evolved towards minimally invasive therapy, such as endoscopic and image-guided percutaneous catheter drainage, leaving laparoscopic and open surgical techniques in case of failure of the above-mentioned techniques^[33]. Proper patient selection is essential to decide on the best available technique for each and every patient. Treatment is a complex decision-making procedure, individualized for each patient, encompassing a multidisciplinary approach according to local expertise with endoscopic, percutaneous, or surgical techniques. A recent review has reported short-term clinical success rates of 85% for endoscopic drainage, 83% for surgical techniques and 67% for percutaneous drainage, and complication rates were 16%, 45% and 34% for endoscopic, surgical and percutaneous approaches, respectively^[38].

Ductal anatomical abnormalities are more frequently encountered after severe acute pancreatitis. Although there is a lack of epidemiological data, the incidence of disconnected pancreatic duct syndrome is 10%-30% among patients with severe acute pancreatitis^[39]. According to the revised Atlanta definition of pseudocysts, communication of a pseudocyst with the main duct occurs rarely^[2], while previous studies report that 25%-58% of pseudocysts may communicate with the pancreatic duct^[40]. Displaying such a communication or a disconnected pancreatic duct syndrome is essential in order to establish proper management. ERCP might be considered to further delineate anatomy, but it is not necessary when high-quality cross-sectional imaging (MRCP) is available^[41]. The concurrent use of secretin has improved the diagnostic yield of MRCP and it is suggested to be valuable in assessing ductal continuity^[42]. The ready availability of MRCP renders it the preferred imaging study. ERCP is an invasive method with the risk of contaminating the pseudocyst^[33]. Once ERCP is carried out preoperatively, it should be done in close proximity to interventions to reduce the risk of contaminating the pseudocyst^[43].

OUTCOMES AFTER DIFFERENT MANAGEMENT PLANS

Schematic presentation of the alternative treatment options and their outcomes is depicted in Figure 1.

Patients unfit for surgery

Conservative treatment: Conservatively treated patients after recovering from an acute episode of biliary pancreatitis have a significant risk of developing recurrent biliary events^[7]. Studies have reported recurrence rates of biliary pancreatitis between 18%

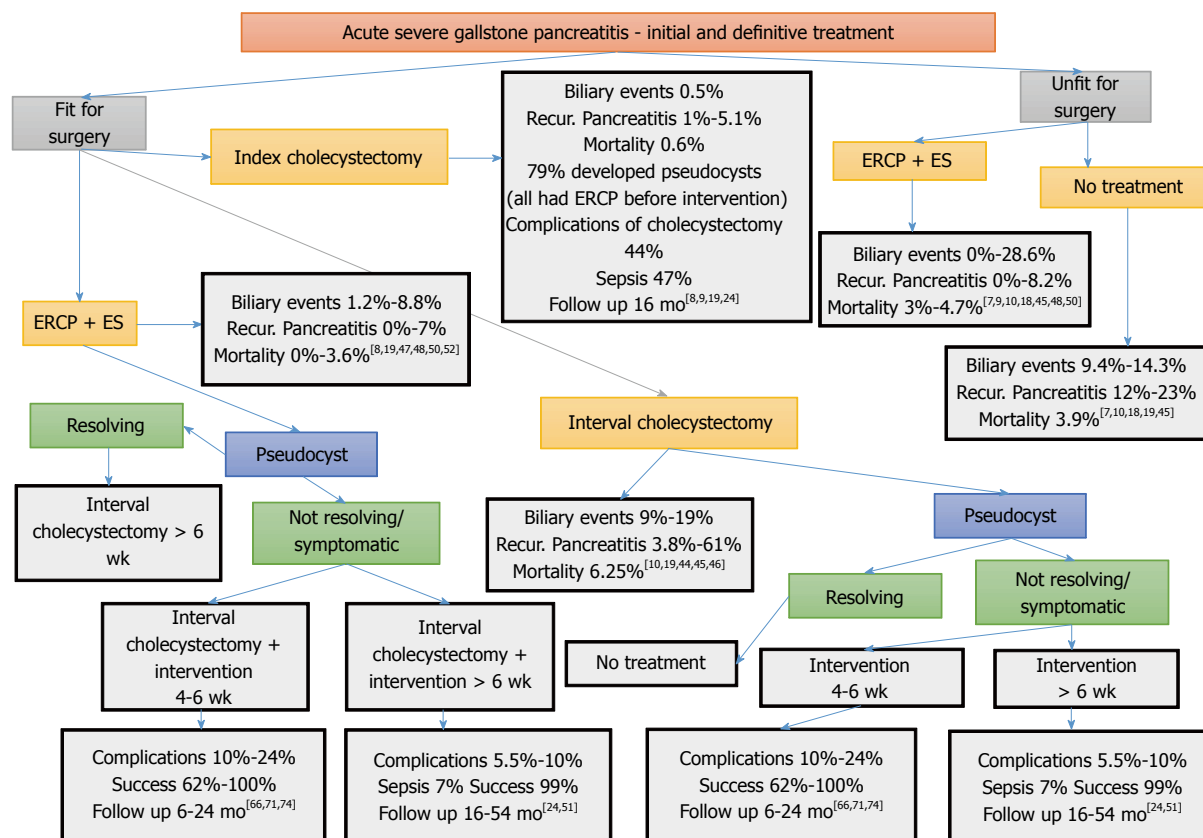


Figure 1 Schematic presentation of alternative treatment options and outcomes for management of acute severe biliary pancreatitis. ERCP: Endoscopic retrograde cholangiopancreatography; ES: Endoscopic sphincterotomy.

and 61% while patients are waiting to undergo cholecystectomy when conservatively treated at index admission^[19,44]. Around 4%-50% of cases of recurrent biliary pancreatitis can be severe^[19]. In a recent retrospective study including 5079 patients, recurrent biliary pancreatitis occurred in 23% of patients, with a median time to the next episode of about 186 d, if no intervention was performed^[10]. Other biliary events may have occurred in patients who did not receive cholecystectomy after having recovered from an acute episode of biliary pancreatitis, including biliary colic (5.2%-11.8%), acute cholecystitis (1%-5.6%) and cholangitis (0.8%-7%)^[19,45-48]. ERCP and sphincterotomy do not protect against the risk of biliary disease^[49].

ERCP and sphincterotomy: Patients receiving ERCP and sphincterotomy alone have a risk of developing recurrent pancreatitis ranging from 0% to 8.2%^[7,9,10,18,45,48,50] and biliary disease ranging from 0% to 28.6%^[10,18,45,50]. Therefore, ERCP and sphincterotomy as definitive treatment in acute severe biliary pancreatitis is at present recommended in patients unfit for cholecystectomy, with severe comorbidity or in necrotizing pancreatitis^[5,6,47,48].

Patients fit for surgery

Index cholecystectomy: The British Society of Gastroenterology guidelines underline that, in patients

with severe acute pancreatitis, cholecystectomy should be postponed until all signs of systemic disorders have resolved^[23]. Furthermore, the American College of Gastroenterology guidelines state that, in order to avoid contamination in patients with necrotizing biliary acute pancreatitis, cholecystectomy should be delayed until the inflammatory process has subsided, and fluid collections have resolved or become stable^[5].

An increased incidence of contaminated collections is observed when performing early cholecystectomy after moderate to severe pancreatitis^[24]. Cholecystectomy is typically delayed in patients with severe acute pancreatitis until a later time during index admission or after discharge, even weeks or months after the pancreatitis episode, or, if pancreatic necrosis is present, cholecystectomy can be performed along with necrosectomy^[13,21,22,51].

Initial ERCP and sphincterotomy: In patients who did not undergo cholecystectomy, the risk of recurrent pancreatitis was significant, 8.2% in patients who had ERCP and 17.1% in patients with no intervention, after a median follow-up of 2.3 years^[7]. Despite the fact that ERCP substantially prevents recurrent pancreatitis, it does not prevent acute cholecystitis and biliary colic^[19]. The value of ERCP in patients with acute biliary pancreatitis and concomitant cholangitis is well recognized^[5,6,25,33], whereas, the role and timing

of ERCP with sphincterotomy in patients with predicted severe biliary pancreatitis without cholangitis or a high suspicion of a persistent common bile duct stone remains subject to debate^[33].

A recurrent rate of acute biliary pancreatitis, between 0% and 7% was observed in patients who had ERCP and sphincterotomy at index admission but did not receive cholecystectomy^[8,19,47,48,50,52]. The risk of recurrent pancreatitis was reduced after sphincterotomy^[7,9,19,48,49]. There is ample evidence to support the belief that sphincterotomy at index admission with interval cholecystectomy is a safe and accurate practice and is considered an alternative to index cholecystectomy in patients with severe biliary pancreatitis^[13,21,47,48,50,53]. In a retrospective study, no readmissions with recurrent acute pancreatitis or biliary symptoms were observed in patients with severe biliary pancreatitis that had ERCP and sphincterotomy as definitive treatment in patients not fit for cholecystectomy, during median follow-up of 3.1 years^[48]. A second retrospective study including patients with moderately severe pancreatitis reported that, after ERCP, no episodes of recurrent pancreatitis were detected while waiting for interval cholecystectomy^[47]. Interval laparoscopic cholecystectomy has the potential of recurrent biliary events and additional hospital stay related to a second admission^[22,45]. Wilson *et al*^[22] concluded that patients with moderate to severe acute biliary pancreatitis should undergo interval cholecystectomy at a later time, weeks or months after recovering from the initial episode, depending on the patients' clinical condition, provided the patient underwent ERCP and sphincterotomy at index admission. A large population-based study provided evidence that cholecystectomy and ERCP at index admission were associated with significantly reduced 12-mo readmission rates for acute biliary pancreatitis^[8].

Initial interval cholecystectomy: Patients with no fluid collections can undergo cholecystectomy after the inflammatory process has subsided and the clinical condition has improved^[13]. Laparoscopic cholecystectomy at index admission is technically demanding and, due to the inflammatory process, it is frequently converted to an open procedure. Interval cholecystectomy probably increases the success rate of laparoscopic cholecystectomy and makes it safer for patients with decreased morbidity^[21,24]. Although interval cholecystectomy allows the inflammatory response to resolve, it has been demonstrated that it cannot lessen severe adhesions, elude difficult dissection of the cystic duct and artery in Calot's triangle, or avoid bleeding, thus resulting in a prolonged operating time^[54].

As pseudocyst formation may occur in patients recovering from an acute episode of severe biliary pancreatitis, cholecystectomy can be combined with procedures for internal drainage of pseudocysts if they

do not resolve after 6 wk^[13], thus avoiding a possible second procedure to drain a pseudocyst^[48]. Timing of interval cholecystectomy varies among studies and it has been reported that patients with severe biliary pancreatitis underwent interval cholecystectomy within 6 mo^[48]. In a multicenter study including 523 patients with biliary pancreatitis, fewer operative complications during cholecystectomy were observed between 4 and 7 wk after discharge, and higher at index admission up to 2 wk after discharge^[55]. Since delaying surgery further than 2 wk after discharge has no apparent unfavorable effect, and delaying definitive management after 12 wk has no prominent advantage, definitive management within 3 mo of admission may decrease recurrent biliary events, readmission rates and operative risk^[55]. In patients recovering from an episode of acute pancreatitis and a small pseudocyst with mild symptoms, cholecystectomy can be delayed for a further 3 mo, since spontaneous resolution of the pseudocyst may still occur^[56].

Management of pseudocysts and walled-off necrosis according to management plans:

Most of the fluid collections generally resolve spontaneously without the need for further intervention, but 5%-16% of patients with severe acute pancreatitis will develop a pseudocyst > 4 wk after onset of pancreatitis^[57,58]. The prevalence in biliary pancreatitis is 6%-8%^[59]. A pseudocyst will also develop in 8% of patients who have undergone necrosectomy^[60]. In a recent prospective multicenter study including 302 patients with acute pancreatitis, pancreatic pseudocysts developed in 6.3% of patients after 4-6 wk. A decrease in size or spontaneous resolution of pseudocysts was observed in an elevated percentage of patients that increased to 84.2% with conservative management^[58].

Italian Association for the study of the Pancreas consensus guidelines on severe acute pancreatitis point out that size < 4 cm is a predictor of spontaneous resolution^[33]. Furthermore, a prospective study including 369 patients found that prognostic factors for spontaneous resolution of pancreatic pseudocysts after an episode of acute pancreatitis were mild or presented no symptoms and a maximum pseudocyst diameter < 4 cm^[56]. A large pseudocyst size itself does not necessitate drainage, although pseudocysts > 6 cm persisting for > 6 wk tend to be symptomatic and have a low likelihood of resolution^[11,12]. The American College of Radiology appropriateness criteria in 2009 recommend drainage of complicated pseudocysts \geq 5 cm that are rapidly enlarging, obstructing, and infected^[17]. According to the American College of Gastroenterology guidelines of 2013, asymptomatic pseudocysts and pancreatic or extrapancreatic necrosis regardless of size, location, or extension do not require intervention^[5]. The asymptomatic patient is a controversial issue. A wait-and-see policy can be adopted in patients with asymptomatic pseudocysts or minimally symptomatic patients, even after the 6 wk

that are required for maturation of a pseudocyst^[61,62]. Indications for intervention are symptomatic pseudocysts with persistent pain, nausea and vomiting, or complications, such as infection, gastric or duodenal outlet obstruction, biliary obstruction, rupture and rapidly enlarging cysts^[16,63].

Integrity of the main pancreatic duct and awareness of the available techniques help in applying the most appropriate intervention, among endoscopic, surgical and image-guided percutaneous techniques. Pancreatic ductal anatomy is clearly associated with the outcome of pseudocysts managed by percutaneous drainage, with favorable outcomes in patients with normal ducts, and satisfactory outcomes in patients with stricture but no cyst-duct communication^[41]. Percutaneous drainage is associated with a high recurrence rate and risk of secondary infection and formation of a pancreatic fistula; thus, it is best applied to infected pseudocysts or patients not suitable for an endoscopic or surgical procedure^[64]. Percutaneous drainage alone is associated with therapeutic rates ranging from 14% to 32%, therefore it is usually performed as a temporary measure before endoscopic or surgical management^[17,65].

Successful drainage can be achieved with the endoscopic approach in 82%-100% of pancreatic pseudocysts, with complications ranging from 5% to 16% and recurrence rates up to 18%^[16,66]. Due to high success rates and low complications rates, the endoscopic approach emerges as the most efficient method. Transpapillary drainage has been used for pancreatic pseudocysts communicating with the main pancreatic duct but it is associated with ERCP-related complications, contamination of the pseudocyst, and insufficient drainage of large cysts^[16,61]. Pseudocysts > 4 cm require transmural drainage, preferably with EUS guidance but conventional endoscopy also offers good results^[61]. Transmural drainage has gradually become the preferred therapeutic approach for managing pseudocysts, including the advantages of cystogastrostomy or cystoduodenostomy (internal drainage)^[16]. A recent multicenter retrospective study found that transpapillary drainage of pseudocysts in patients undergoing EUS-guided transmural drainage added no benefit to outcomes and adversely affected resolution of the pseudocyst^[67].

Transmural drainage can be carried out either by direct endoscopy or by EUS guidance. Endoscopy by EUS guidance is increasingly used in particular for pseudocysts in which there is no definitive luminal bulge, or when managing patients with portal hypertension or coagulopathy^[68]. A recent systematic review reported mean technical and clinical success rates of 97% and 90%, respectively, mean overall recurrence rate of 8%, and overall complication rate of 17% for EUS-guided drainage^[69]. A meta-analysis comparing EUS-guided drainage with conventional transmural drainage for pseudocyst found that technical success rate was significantly higher for EUS-guided drainage

but not superior to conventional transmural drainage in terms of short- and long-term success, and overall complications were similar in both groups^[70]. A randomized trial comparing patients undergoing EUS-guided drainage with conventional transmural drainage for pseudocysts found a technical success rate of 100% and 33%, respectively^[71]. A second randomized trial also found a higher technical success rate for patients undergoing EUS-guided drainage than for conventional endoscopic drainage (94% vs 72%)^[72]. While high clinical success rates have been reported when draining pseudocysts with endoscopic procedures, clinical success rates for walled-off necrosis are relatively poor due to the presence of solid material. Multiple transluminal gateway treatment is suggested for walled-off necrosis, thus avoiding the need for surgery or endoscopic necrosectomy^[73] or other more complex procedures. Endoscopic procedures for pseudocyst drainage are technically feasible only if access to the pseudocyst through the gastric or duodenal wall can be achieved; at present performance of an endoscopic cystjejunostomy is not possible. Patients requiring more complex management of their pseudocyst are not candidates for endoscopic procedures. Another limitation of the endoscopic approach is the inability to perform an additional cholecystectomy when necessary, as patients with biliary pancreatitis require open or laparoscopic cholecystectomy^[4].

Surgical cystogastrostomy or cystojejunostomy has been the traditional approach for pseudocyst management and is still the preferred treatment in most centers with a success rate of 94%-99%^[4,74]. Open or laparoscopic surgical drainage should be applied after failure of endoscopic methods, for recurrence after a successful endoscopic drainage, and in patients who do not meet the criteria for endoscopic or percutaneous drainage^[38]. Moreover, percutaneous and endoscopic techniques, if not therapeutic, can serve as a bridge to surgery and improve patients' local and general condition^[24]. Laparoscopy is a minimally invasive method that achieves sufficient internal drainage and debridement of necrotic tissue, with good results and minimal morbidity^[75]. In a large series on laparoscopic cystogastrostomy, the authors conclude that laparoscopy has a significant role in the surgical management of pseudocysts, with favorable outcomes^[51]. A retrospective study and a randomized trial, both by Varadarajulu *et al.*^[74,76], comparing EUS-guided drainage with open surgical cystogastrostomy found no significant difference in pseudocyst recurrence between the two groups. A drawback of these studies is the implementation of open surgery.

In patients with pseudocysts who have recovered from an acute episode of moderate to severe biliary pancreatitis, interval cholecystectomy should be delayed until the pseudocyst resolves. If it persists for > 6 wk, operative pseudocyst drainage can be performed safely at this time with concurrent cholecystectomy, thus minimizing the risk for a second inter-

ventional procedure^[24].

CONCLUSION

The following alternatives are feasible for the treatment of patients fit for surgery: index cholecystectomy, interval cholecystectomy, ERCP and endoscopic sphincterotomy followed by delayed cholecystectomy. Cholecystectomy is an essential part of dealing with patients with severe biliary pancreatitis. Current guidelines recommend delaying cholecystectomy until resolution of inflammatory process has occurred, usually not earlier than 6 wk after onset of acute pancreatitis. It seems that ERCP and endoscopic sphincterotomy protect patients better than interval cholecystectomy for recurrent pancreatitis and biliary episodes, while at the same time providing more time for the potential pseudocyst either to resolve or become stable for intervention. The optimal time point for applying any available pseudocyst treatment modality is > 6 wk. Recent trends in management of pseudocysts involve minimally invasive therapeutic techniques, but surgical approaches are still frequently used, especially if combined with cholecystectomy at the appropriate time. EUS and MRCP with secretin are reliable tools for tailored patient therapy. In patients unfit for surgery, the application of ERCP and endoscopic sphincterotomy has better results than conservative management.

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Recent advances in orally administered cell-specific nanotherapeutics for inflammatory bowel disease

Xiao-Ying Si, Didier Merlin, Bo Xiao

Xiao-Ying Si, Bo Xiao, Institute for Clean Energy and Advanced Materials, Faculty of Materials and Energy, Southwest University, Chongqing 400715, China

Didier Merlin, Bo Xiao, Institute for Biomedical Sciences, Center for Diagnostics and Therapeutics, Georgia State University, Atlanta, GA 30302, United States

Didier Merlin, Atlanta Veterans Affairs Medical Center, Decatur, GA 30033, United States

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Correspondence to: Bo Xiao, PhD, Institute for Clean Energy and Advanced Materials, Faculty of Materials and Energy, Southwest University, Tiansheng Road No. 2, Chongqing 400715, China. hustboxiao@gmail.com
Telephone: +86-23-68254762
Fax: +86-23-68254969

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Abstract

Inflammatory bowel disease (IBD) is a chronic relapsing disease in gastrointestinal tract. Conventional medications lack the efficacy to offer complete remission in IBD therapy, and usually associate with serious side effects. Recent studies indicated that nanoparticle-based nanotherapeutics may offer precise and safe alternative to conventional medications *via* enhanced targeting, sustained drug release, and decreased adverse effects. Here, we reviewed orally cell-specific nanotherapeutics developed in recent years. In addition, the various obstacles for oral drug delivery are also reviewed in this manuscript. Orally administrated cell-specific nanotherapeutics is expected to become a novel therapeutic approach for IBD treatment.

Key words: Oral administration; Nanotherapeutic; Cell-specificity; Inflammatory bowel disease

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Core tip: Inflammatory bowel disease includes Crohn's disease and ulcerative colitis. Nanotherapeutics may outperform conventional medications *via* the targeted drug delivery, sustained drug release, and decreased adverse effect. The main purpose of this review is to offer an update of efficacy of the orally administrated cell-specific nanotherapeutics that have been developed recently.

Si XY, Merlin D, Xiao B. Recent advances in orally administered cell-specific nanotherapeutics for inflammatory bowel disease. *World J Gastroenterol* 2016; 22(34): 7718-7726 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i34/7718.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i34.7718>

INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic relapsing gastrointestinal (GI) disorder with no permanent cure. It mainly includes Crohn's disease (CD) and ulcerative colitis (UC), and affects millions of patients worldwide. After 30 years of living with this disease, 8% of CD and 18%-20% of UC patients develop colitis-associated colon cancer, which is the third most common malignancy and one of the leading causes of cancer mortality^[1]. Although the etiology of IBD remains largely unknown, a large amount of researches over the last decades demonstrated that the individual's genetic susceptibility, intestinal microbiota, and immune responses are all involved in the pathogenesis of IBD^[2]. Conventionally, IBD is treated by daily administration of high doses of anti-inflammatory or immunosuppressive drugs. Some of these treatments are effective in controlling inflammation. However, their applications have been restricted by problems with long-term efficacy and safety issues. For example, as for corticosteroids, a total daily dose over 20 mg of prednisolone for more than 2 wk is associated with an obvious increased risk of infections^[3].

Recently, nanotherapeutics have been recognized as a promising strategy which can potentially revolutionize disease diagnostics and treatments. They offer significant advantages over traditional approaches because of their nanometer scale dimension, targeted drug delivery capacity, controlled drug release, and decreased adverse effects^[4,5]. Most importantly, nanotherapeutics have been found to confer similar or even better therapeutic impacts at lower drug concentrations than their conventional counterparts^[6]. It was reported that oral administration has been considered as the most convenient approach for colitis therapy-related drug delivery, as it avoids the pain and discomfort associated with injections, minimizes the potential for contamination, and is applicable for a self-medication that can be fully controlled by patients^[7]. Accordingly, orally targeted nanotherapeutics have been developed.

The challenges for oral drug delivery are to ensure drug formulations to remain stable in the GI tract, transport adequate amount of active drugs to the specific sites, minimize systemic absorption of the drugs, and lower the risk of adverse side effects^[8]. The earliest nanotherapeutics designed for IBD-targeted therapy are based on the physiological features that are particular to colon to trigger drug release^[9]. However, physiological conditions can differ among patients and

at various stages of IBD, making it very difficult to attain sufficient therapeutic efficiency. Parallel breakthroughs in the understanding of the molecular pathophysiology of IBD and the development of intelligent NPs offer tremendous promise for IBD therapy^[10]. In this review, we focus on novel therapeutic approaches using orally targeted nanotherapeutics and their challenges in GI tract.

OBSTACLES FOR ORALLY NANOTHERAPEUTICS

GI tract

After oral administration, NP-based nanotherapeutics pass through the esophagus, the stomach, the small intestine and the colon, successively. The pH in the passage ranges from strongly acidic in the stomach (pH 1.5-1.9), to almost neutral in the small intestine, and then slightly acidic (pH 5-7) in the colon^[11]. Therefore, NPs have to be stable over a wide pH range. In addition, they also encounter digestive enzymes in stomach (e.g., pancreatic enzymes), bicarbonate and bile salts in the small intestine, as well as abundant microbial population in colon. All of these contents in the GI tract can destabilize NPs and further reduce the effectiveness of their loaded drugs^[12,13]. Besides, the semi-solid contents in colonic lumen prevent NPs from diffusing into the inflamed sites.

Mucus

The mucus on the surface of colon epithelial layer is highly viscoelastic and adhesive, and forms a thick layer ($830 \pm 110 \mu\text{m}$)^[14]. It is mainly composed of mucins and lipids, and acts to trap and remove bacteria, viruses, and foreign matters^[15]. In healthy colon, there is a continuous mucus which has two layers of sub-structures: the outer is a loosely adherent layer for bacterial adhesion; while the inner is a tightly adherent layer, normally sterile. In colon tissue with IBD, there is a marked increase in bacteria associated with colonic adherent mucus layer^[12,16].

Maisel *et al.*^[17] developed an unmodified NPs that were mucoadhesive (mucoadhesive particles, MAP), and a PEG-coated NPs which were non-mucoadhesive (mucus-penetrating particles, MPP). In comparison to MAP, MPP tended to penetrate in the GI tract, including colitis tissue. In addition, Ijssennagger *et al.*^[18] demonstrated that hydrogen sulfide, mainly produced by sulfate-reducing bacteria, reduced disulfide bonds presented in the mucus network, thereby breaking the mucus barrier. Reduction of disulfide bonds in the gut lumen might represent an exciting method for the penetration of NPs to mucosa.

Epithelial enhanced permeability and retention effect

Inflamed colon is associated with disruption of the intestinal epithelial layer and accumulation of immune cells, leading to the loss of barrier function and

increased epithelial permeability^[19]. NPs are likely to penetrate into the gaps among epithelial cells, thus increasing the local drug concentration and exerting therapeutic effects there. This phenomenon is called "epithelial enhanced permeability and retention" effect^[20,21]. This effect is size-dependent, showing a maximum efficacy in the nano range. Furthermore, in comparison to free drugs in solution or suspension, drug-loaded NPs were shown to accumulate to a greater extent in inflamed tissues and prolong therapeutic effects^[20]. Additionally, anti-inflammatory chemical drugs loaded into NPs also showed enhanced cellular uptake by cells in colitis tissues, due to protection of the drugs from efflux systems and mucosal metabolism^[22].

Cellular uptake

NPs generally undergo cellular internalization by paracellular transport or endocytosis into epithelial cells in the GI tract. In the context of cellular uptake by inflamed colon, endocytosis might be the main NP cellular uptake approach. Endocytosis can be triggered by the interaction between the surface moieties of NPs and the specific receptors, which are highly over-expressed on the surface of IBD therapy-related key cells in colitis tissue. Critically, the internalization and transportation of NPs may be enhanced by the specific targeting to these receptors^[23].

Endosomal escape

NPs are entrapped in endosomes after internalization into cells. Subsequently, proton pump, an integral membrane protein, moves protons across the endosome membrane, inducing the pH continuously decrease from 7.2-7.4 to 4.5-5.0^[24]. To avoid their degradation in endosome, many therapeutics (*e.g.*, protein, plasmid DNA and siRNA) have to escape from the endosome into the cytosol, where they can associate their targets^[25].

To induce efficient endosomal escape, a common strategy is to introduce chemical groups with proton-sponge effect to NPs. Our group previously synthesized a mannosylated bio-reducible cationic polymer (PPM) and further spontaneously assembled NPs with siRNA assisted by sodium triphosphate (TPP). In these TPP-PPM/siRNA NPs, the abundant primary and tertiary amine groups in PPM can promote endosomal escape efficiently^[26].

Nuclear localization

After escaping from the endosome, drugs often have to enter certain organelles in order to exert their functions there. Nuclear entry is a prerequisite for some drugs, such as inhibitors of transcription or the cell cycle^[27]. For instance, plasmid DNA must be transported into the nucleus. Otherwise, transcription cannot occur^[28].

Wang *et al.*^[29] synthesized a series of *N*-terminal

stearylated nuclear localization signals, and their further studies showed that such vectors could effectively deliver plasmid DNA into nuclei. The maximum transfection efficiency of these vectors was 80% of that of jetPEITM.

NANTHERAPEUTICS FOR IBD

Conventional NPs

Polyester NPs: Polylactic-co-glycolic acid (PLGA) and polylactic acid (PLA), FDA-approved biodegradable polyesters, have the capacity to encapsulate hydrophilic or hydrophobic drugs to form NPs. Thus, they have been commonly used as drug carrier materials^[5,30,31].

Mahajan *et al.*^[32] loaded mesalamine into PLGA NPs, and they further administered them once a day to rats with colitis through oral administration or intracolonic administration. It was found that these NPs exerted much higher efficiency in mitigating colitis, in comparison to the free drug in suspension. In addition, the researchers also demonstrated that the mesalamine-loaded PLGA NPs showed selective adherence and enhanced drug accumulation into colitis tissues.

Silicon NPs: Silicon NPs have been widely used as drug vectors since they possess a range of beneficial features for drug delivery, including well-controlled size and size distribution, easily surface functionalization, and negative cytotoxicity, as well as large surface area and pore volume that facilitate drugs to be encapsulated^[33].

Moulari *et al.*^[34] prepared 5-aminosalicylic acid-loaded silica NPs (around 140 nm), which showed 6-fold better adherence to inflamed colon than tissues from the healthy control mice after oral administration. In trinitrobenzene sulfonic acid (TNBS)-induced colitis mouse model, the NPs tended to accumulate in inflamed sites, and exerted excellent therapeutic efficacy in terms of clinical activity score and myeloperoxidase activity at lower drug doses than those applied in conventional delivery.

COMPLEX NPS

pH-sensitive NPs: pH-sensitive NPs take advantage of the pH differences in different regions of the GI tract^[35]. The pH in the terminal ileum and colon is generally higher than that in any other regions of the GI tract. One of the simplest ways to modify dosage forms for pH-dependent drug delivery is to coat them with pH-sensitive biocompatible polymers.

Ali *et al.*^[36] showed that budesonide-loaded PLGA NPs that were further coated with Eudragit S100 (Figure 1A upper) significantly alleviated inflammation in the dextran sulfate sodium (DSS)-, TNBS- and oxazolone-induced mouse colitis models. A pH-sensitive NPs can

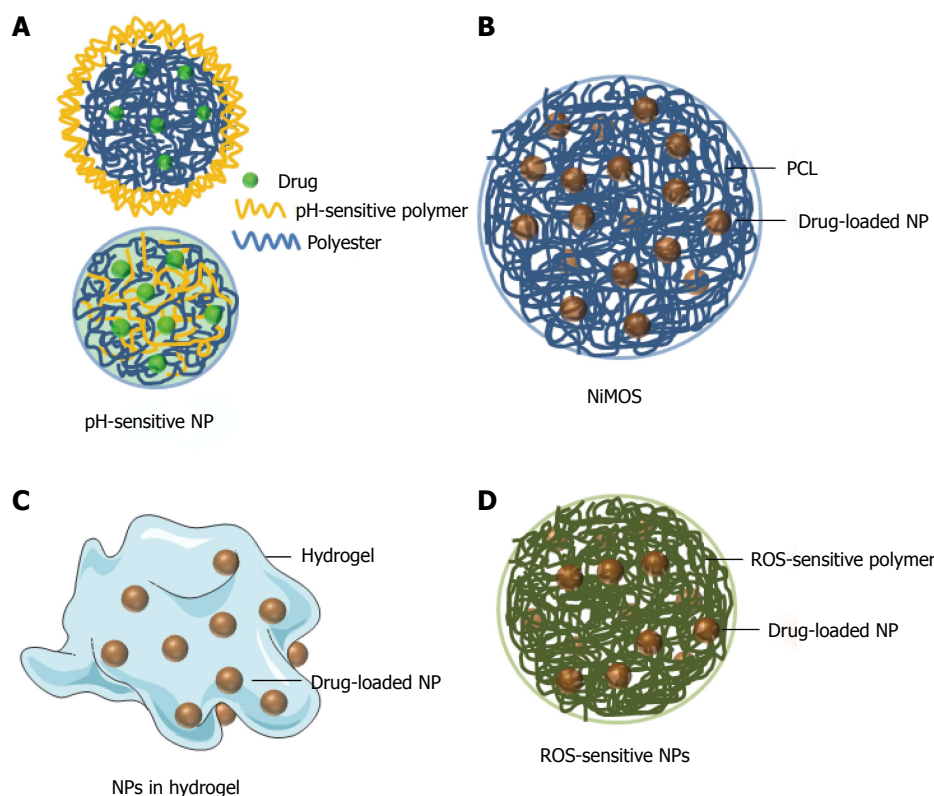


Figure 1 Various types of complex NPs for oral drug delivery. A: pH-sensitive NP; B: NiMOS; C: NPs in hydrogel; D: ROS-sensitive NP.

also be produced by mixing a pH-sensitive polymer and a non-pH-sensitive polymer in the NP fabrication process (Figure 1A lower). Belouqui *et al.*^[37] found that curcumin-loaded Eudragit S100/PLGA NPs showed obvious pH sensitivity profiles and efficiently decrease the expression/secretion of TNF- α in lipopolysaccharide-activated macrophages. Critically, oral administration of these pH-sensitive NPs significantly reduce neutrophil infiltration and TNF- α secretion in DSS-induced colitis mice. Additionally, histological studies revealed that the treatment mice exhibited almost the same colonic structure morphology as that observed in the healthy control group.

NP-in-microparticle: Recently, Amiji's group developed a colon-targeted drug formulation named the NP-in-microparticle (MP) oral drug delivery system (NiMOS), as demonstrated in Figure 1B^[38]. A multi-compartmental NiMOS drug delivery system consists of gelatin nanoparticles encapsulated within poly- ϵ -caprolactone (PCL) MPs^[39]. The MP matrix inhibits protein/enzyme degradation, and thus avoids the harsh environment of the GI tract capable of destroying the embedded NPs. When NiMOS reaches the colon, lipases degrade the PCL coating and the encapsulated NPs will be released to colon, which would become available for the uptake by colonic cells^[40-43].

Kriegel *et al.*^[43] prepared TNF- α siRNA (siTNF)-loaded gelatin NPs, and further encapsulated them in PCL matrix to yield NiMOSs. The animal experiment

results based on a DSS-induced colitis mouse model demonstrated that the above NiMOSs remarkably suppressed the expression levels of TNF- α and other pro-inflammatory cytokines (*e.g.*, IL-1 β , IFN- γ , and monocyte chemoattractant protein-1), and increased the body weight and colon length, in comparison to DSS-treated control mice. These results indicated that siTNF-loaded NiMOS could be a valuable therapeutic option for IBD patients.

NPs in hydrogel: Hydrogels are highly absorbent polymeric networks, and they can contain over 90% water. Chitosan and alginate are biocompatible and biodegradable polysaccharides, and used to prepare oral hydrogel by Merlin's group^[44]. This chitosan/alginate hydrogel was sensitive to the colonic pH and could also be degraded by colonic enzymes. Thus it would collapse when they arrived at colon.

Laroui *et al.*^[45] embedded Lysine-proline-valine (KPV)-containing PLA NPs in this hydrogel (Figure 1C). Under its protection, NPs were able to pass through the stomach and upper small intestine, and were released in the inflamed colon. Using this improved NPs in hydrogel system, a 12000-fold lower dose was sufficient to ameliorate mucosal inflammation in mice subjected to acute DSS-induced colitis, in comparison to free KPV in solution.

Reactive oxygen species-sensitive NPs: IBD is accompanied by abnormally high reactive oxygen species (ROS) level in the GI tract, especially in the

Table 1 Ligands for orally targeted drug delivery in inflammatory bowel disease

Ligand	Delivery system	Effect	Ref.
Lectin	PLGA	Exhibited a much higher binding and selectivity to inflamed tissue compared to plain NPs	[54]
TfR antibodies	Liposomes	Exhibited mucopenetration and a 4-fold increase in uptake by inflamed colon tissues	[58]
CD98 antibodies	PEG-urocanic acid-chitosan	Approximately 24% of colonic macrophages were found to have taken up the targeted NPs within 12 h of administration	[66]
Mannose	Branched polyethylenimine	29.5% of the NPs were internalized by colon macrophages	[26]
Galactose	Trimethyl chitosan-cysteine	Cellular uptake in activated macrophages was significantly higher for Galactose trimethyl chitosan-cysteine/TPP NPs compared to trimethyl chitosan-cysteine/TPP NPs	[75]
F4/80 Ab Fab'	Poly(lactic acid)-poly(ethylene glycol) block copolymer	Improved DSS-induced colitis in vivo, and higher therapeutic efficacy was obtained using Fab'-bearing NPs compared to non-conjugated NPs	[77]
Amphiphilic hyaluronic acid	Decylamine	Budesonide loaded HANPs demonstrated higher anti-inflammatory effect on IL-8 and TNF- α secretion in inflamed cell model compared to the same dose of free drug	[78]

TfR: Transferrin receptor; PLGA: Polylactic-co-glycolic acid.

inflamed areas^[46]. The excessive ROS can degrade the extracellular matrix and injure tissues^[47]. Based on this fact, ROS-sensitive NPs (Figure 1D) are supposed to specifically release loaded drugs to inflamed colon or scavenge ROS.

Wilson *et al.*^[48] synthesized poly(1,4-phenyleneacetone dimethylenethioketal) (PPADT), which is a novel ROS-sensitive polymer. PPADT were further used to encapsulate siTNF/DOTAP complexes to generate thioketal NPs (TKNs). In a DSS-induced colitis mouse model, the mRNA expression levels of TNF- α and several other pro-inflammatory cytokines (IL-1, IL-6 and IFN γ) were significantly decreased and DSS-induced acute colitis was efficiently ameliorated after 5 days of oral administration of TKNs (0.23 mg siRNA/kg body weight)^[49].

It was also reported that antioxidant compounds and free radical scavengers improved colitis^[50,51]. Vong *et al.*^[52] synthesized a novel antioxidative nitroxide radical-containing NP (RNP^o) with a diameter of 40 nm. The further mice experiments indicated that the extent of inflammatory reactions and the severity of colitis have been relieved after oral administration of RNP^o.

LIGAND-MEDIATED TARGETED DRUG DELIVERY

To further reduce side effects and increase the drug concentration at inflamed sites, researchers have sought to induce active targeting^[53]. The interactions between targeting ligands on the NPs surface and specific receptors over-expressed at inflamed sites are expected to improve the bioadhesion and the internalization of NPs to specific cells. Various ligand-mediated targeted drug delivery systems are compared in Table 1, and their targeted drug delivery process is shown in Figure 2.

Lectin

Lectin is a type of naturally occurring protein that is highly specific for carbohydrate residues. It has been widely used as a ligand facilitating colon-specific drug delivery.

Moulari *et al.*^[54] prepared two types of lectin-functionalized NPs that were peanut (PNA)-functionalized NPs and wheat germ (WGA)-functionalized NPs. *Ex vivo* quantitative adhesion analyses showed that lectin-functionalized NP exhibited a much higher binding and selectivity to inflamed tissue compared to plain NP. In terms of therapeutic efficacy, all glucocorticoid containing formulations revealed an enhanced therapeutic effect with lectin functionalization, especially by the PNA-NP compared to plain NP.

Transferrin receptor antibody

Healthy colon tissue has low expression level of transferrin receptor (TfR), whereas inflamed colon overexpresses TfR. Its elevated expression was detected in both basolateral and apical membranes of enterocytes from the colon biopsies of IBD patients and rats with colitis^[55]. In addition, TfR levels are also found to be elevated in activated immune cells (e.g., lymphocytes and macrophages)^[56,57]. Thus, it is reasonable to speculate that TfR could be used as a targeting receptor for colitis-targeted drug delivery.

Harel *et al.*^[58] investigated the *ex vivo* adhesion capacity of anti-TfR antibody-functionalized immunoliposome to inflamed mucosal tissue^[59]. The results indicated that this liposome exhibited mucopenetration and a 4-fold increase in uptake by colitis tissues from TNBS-induced colitis mice, compared to healthy colon tissues.

CD98 antibody

CD98 is a heterodimeric neutral amino acid transporter, which consists of a heavy chain (CD98hc or SLC3A2)

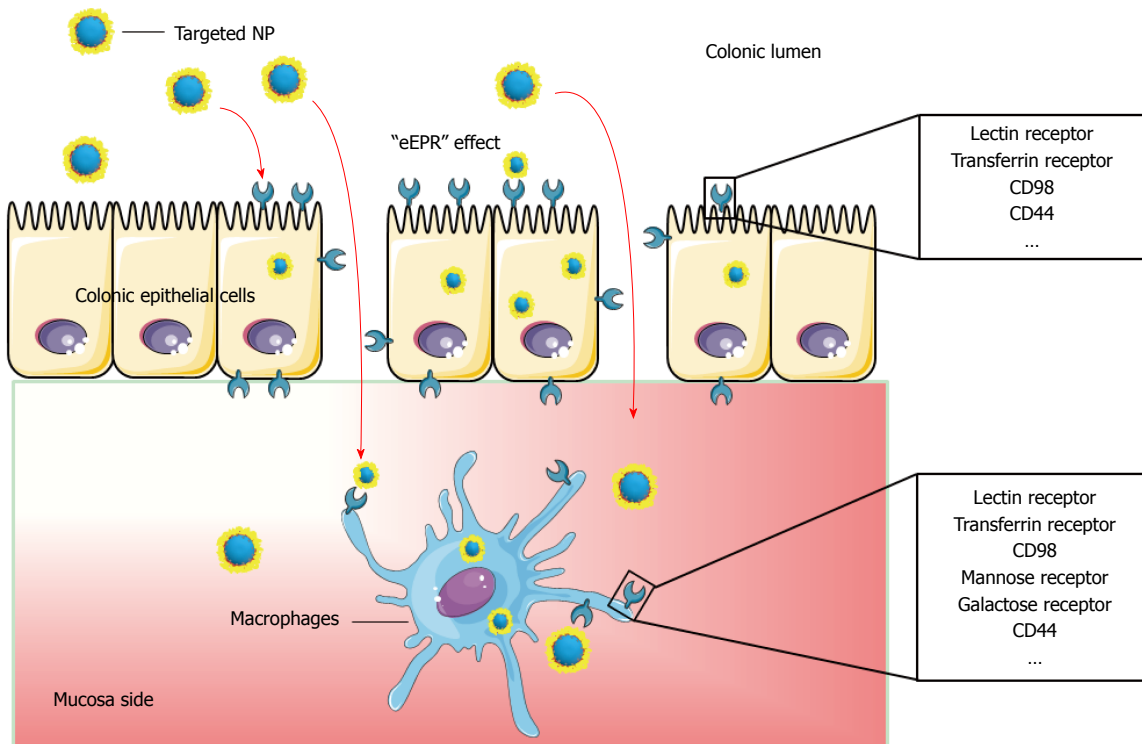


Figure 2 Schematic illustration of cell-specific drug delivery by nanotherapeutics after oral administration.

and one of several versions of the L-type amino acid transporter 1^[60]. In colonic tissues from mice with active colitis, the expression level of CD98 is highly up-regulated at the surface of intestinal B cells, CD4⁺ T cells, and CD8⁺ T cell^[61-63]. Additionally, CD98 is over-expressed in intestinal macrophages^[64] and colonic epithelial cells from mice with colitis^[61,65].

Recently, Xiao *et al.*^[66] fabricated an orally delivered hydrogel that releases CD98 antibody-functionalized NPs with the diameter of around 200 nm. In mice with acute or chronic colitis, orally administrated CD98 antibody-functionalized NPs significantly reduced the CD98 expression level in colitis tissue. Further studies revealed that about 24% of colonic macrophages internalize the targeted NPs within 12 h after oral administration, and the severity of colitis was significantly alleviated compared to the DSS control group.

Mannose

The mannose receptor is a 175-kDa transmembrane protein, and it is exclusively expressed on the surfaces of macrophages^[67]. MRs have high-affinity binding to infectious agents with terminal mannose groups^[61]. Moreover, this receptor can also bind to mannose-functionalized NPs, inducing the subsequent rapid internalization^[68]. Numerous reports demonstrated that the functionalization of mannose on NP surface provides selective macrophage targeting and promotes cell internalization efficiency^[69,70].

Xiao *et al.*^[26] synthesized a mannosylated bioreducible cationic polymer (PPM). It could spontaneously complex with siRNA to form NPs assisted by TPP. The resultant

TPP-PPM/siTNF NPs exhibited obvious macrophage-targeting property. Further *ex vivo* experiment indicated that these NPs markedly inhibited TNF- α secretion in DSS-induced colitis tissues. Importantly, flow cytometry results showed that TPP-PPM/siTNF NPs were efficiently taken up by macrophages (29.5%), whereas there was no significant uptake by the epithelial cells.

Another study presented the similar result by Huang *et al.*^[71]. They fabricated a drug formulation by cationic konjac glucomannan (cKGM), phytigel and an antisense oligonucleotide (ASO) against TNF- α . The unique swelling properties of cKGM induced the spontaneous release of cKGM/ASO NPs from the phytigel matrix to colon lumen. Subsequently, mannose groups can be recognized by MRs that are abundant on the surface of macrophages. The treatment of this oral drug formulation significantly decreased TNF- α expression level and alleviated the symptoms of colitis in DSS-treated mice.

Galactose

It was reported that galactose receptor is highly overexpressed on the surface of activated macrophages^[72,73]. Recently, Zhang *et al.*^[74] fabricated galactosylated trimethyl chitosan-cysteine (GTC) NPs loaded with siRNAs against mitogen-activated protein kinase kinase kinase 4 (MAP4K4), which is a key enzyme of TNF- α production. *In vitro* results showed that the cellular uptake efficiency of GTC/TPP NPs was significantly higher than that of trimethyl chitosan-cysteine/TPP NPs, owing to galactose receptor-mediated endocytosis. Further *in vivo* experiments

demonstrated that oral administration of siMAP4K4-loaded GTC/TPP NPs obviously improved DSS-induced colitis, as characterized by body weight, histology, and myeloperoxidase activity.

F4/80 antibody

F4/80 is one of the vital macrophage-specific markers, and its antibody has been widely applied to separate macrophages^[75,76]. Laroui *et al.*^[77] synthesized poly(lactic acid)-poly(ethylene glycol) block copolymer (PLA-PEG), and then conjugated with a macrophage-specific ligand (F4/80 Ab Fab') *via* maleimide/thiol group-mediated covalent bonding. They further used this polymer to encapsulate TNF- α siRNA (siTNF) to obtain F4/80 Fab'-functionalized siTNF-loaded NPs. Oral administration of siTNF-loaded NPs markedly improved DSS-induced colitis, and exhibited much higher therapeutic efficacy for F4/80 Fab'-functionalized NPs, in comparison to non-functionalized NPs. Further flow cytometry experiments indicated that functionalization of F4/80 Fab' significantly improved their macrophage targeting ability and the endocytosis of these NPs.

Hyaluronic acid

Hyaluronic acid (HA) is a natural anionic polysaccharide, composed of alternating D-glucuronic acid and N-acetyl-D-glucosamine units. It has a high affinity for the CD44 receptor, which is overexpressed on the surface of epithelial cells and activated inflammatory cells in colitis tissue^[78]. Recently, Vafaei *et al.*^[78] synthesized HA-decylamine (DA) by chemical conjugation of DA to the backbone of HA. This amphiphilic HA-DA polymer could then form self-assembled HANPs. FITC-labeled HANPs revealed greater cellular uptake in inflamed Caco-2 BBE cell compared to untreated Caco-2 BBE cell and CD44-negative cell line (NIH3T3). Cytotoxicity test reveals that budesonide (BDS)-loaded HANPs displayed almost no toxicity, indicating HANPs were biocompatible nanocarriers. Importantly, BDS-loaded HANPs exhibited much higher anti-inflammatory effect in inflamed cell model compared to the same dose of free drug.

Overall, these studies demonstrate that active targeting is a very promising approach to enhance the accumulation and uptake of drugs by inflamed tissues.

CONCLUSION

Nanotherapeutic has been widely used as a novel approach for IBD treatment, and is much more effective than traditional drug formulation. Surface functionalization of these nanotherapeutics with targeting moieties (*e.g.*, antibody, monosaccharide, or polysaccharide) can further promote drug accumulation in the IBD therapy-related cells through receptor-mediated endocytosis and decrease the potential adverse effects. As summarized in this review, we provide some novel nano-formulations specific to the organs, tissues, cells, or organelles. We must clearly understand the barriers impeding the

specific delivery of drugs to inflamed colonic tissues or IBD therapy-related key cells. Specifically, there is a need for further investigation of the pathophysiology of IBD (especially in the molecular level) and the development of intelligent NPs.

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Co-existence of non-alcoholic fatty liver disease and inflammatory bowel disease: A review article

Che-Yung Chao, Robert Battat, Alex Al Khoury, Sophie Restellini, Giada Sebastiani, Talat Bessissow

Che-Yung Chao, Robert Battat, Alex Al Khoury, Giada Sebastiani, Talat Bessissow, Division of Gastroenterology, McGill University Health Center, Montreal, QC H3G 1A4, Canada

Sophie Restellini, Division of Gastroenterology and Hepatology, Geneva's University Hospitals and University of Geneva, 1205 Genève, Switzerland

Author contributions: All co-authors have contributed equally to this article.

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Correspondence to: Talat Bessissow, MD, FRCPC, Division of Gastroenterology, McGill University Health Center, 1650 Avenue Cedar C7-200, Montreal, QC H3G 1A4, Canada. talat.bessissow@mcgill.ca
Telephone: +1-514-9341934
Fax: +1-514-9348531

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Abstract

Emerging data have highlighted the co-existence of non-alcoholic fatty liver disease (NAFLD) and inflammatory bowel disease; both of which are increasingly prevalent disorders with significant complications and impact on future health burden. Cross-section observational studies have shown widely variable prevalence rates of co-existing disease, largely due to differences in disease definition and diagnostic tools utilised in the studies. Age, obesity, insulin resistance and other metabolic conditions are common risks factors in observational studies. However, other studies have also suggested a more dominant role of inflammatory bowel disease related factors such as disease activity, duration, steroid use and prior surgical intervention, in the development of NAFLD. This suggests a potentially more complex pathogenesis and relationship between the two diseases which may be contributed by factors including altered intestinal permeability, gut dysbiosis and chronic inflammatory response. Commonly used immunomodulation agents pose potential hepatic toxicity, however no definitive evidence exist linking them to the development of hepatic steatosis, nor are there any data on the impact of therapy and prognosis in patient with co-existent diseases. Further studies are required to assess the impact and establish appropriate screening and management strategies in order to allow early identification, intervention and improve patient outcomes.

Key words: Crohn's disease; Non-alcoholic fatty liver disease; Non-alcoholic steatohepatitis; Ulcerative colitis; Metabolic syndrome

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Core tip: This article reviews the current available literature on issues relating to the co-existence of non-alcoholic fatty liver disease and inflammatory bowel disease with particular focus on the prevalence, risk factors and the clinical implications.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a spectrum of disorders ranging from hepatic steatosis to steatohepatitis (NASH) with associated inflammation and may lead to liver fibrosis along with potential progression to cirrhosis, hepatic failure and hepatocellular carcinoma^[1-3]. Currently, NASH is the second leading etiology of liver disease among adults awaiting liver transplantation in the United States and is predicted to become the leading indication for liver transplant in the near future^[4]. The epidemic of NAFLD in the general population is partly due to the increase in diabetes, dyslipidemia, and obesity. Liver biopsy has long been the gold standard to assess NAFLD and to stage liver fibrosis but this procedure is invasive, costly and not very practical for screening^[5]. Other non-invasive methods to diagnose fatty liver and liver fibrosis have been used including serum biomarkers, ultrasound (US), computed tomography and magnetic resonance imaging. NAFLD is largely asymptomatic until end-stage complications occur. Hence, identification of risk factors, early diagnosis and intervention are pivotal in the management of this common disease.

Inflammatory bowel disease (IBD), consisting of ulcerative colitis (UC) and Crohn's disease (CD), is an increasingly prevalent intestinal disorder with significant co-morbidities. North America has the highest prevalence and incidence rates of IBD worldwide which translate into a significant health care related cost^[6,7]. Elevated transaminases in IBD patients are frequent^[8], with NAFLD being the most common cause^[9]. There are some emerging data suggesting an increase in prevalence of NAFLD in IBD patients compared to the general population, although this is not yet clearly established. Some have attributed this to a general increase of metabolic syndrome (MS) or the increasingly successful IBD therapy in achieving remission and improved nutritional status. However, the pathogenesis of NAFLD in the IBD population may be more complex involving disease-specific risk factors, such as chronic inflammation, drug-induced

hepatotoxicity, steroid exposure, malnutrition and gut dysbiosis^[10,11]. This article examines the prevalence, risk factors and the clinical implications relating to the coexistence of NAFLD in IBD patients.

EPIDEMIOLOGY: PREVELANCE OF NAFLD IN IBD

Cross-sectional studies reported a prevalence of NAFLD in IBD ranging between 6.2% and 40%^[12-14]. A summary of the major studies are provided in Table 1. This discrepancy is largely owed to different definitions and diagnostic tools adopted for NAFLD. Liver fibrosis has been reported in 6.4%-10% of IBD patients, however limited data are available for fibrosis specifically relating to underlying NAFLD^[15,16]. Several studies evaluated NAFLD in IBD using ultrasonography, which has an 85% (95%CI: 79.5%-88.9%) sensitivity and 94% (95%CI: 87.2%-97%) specificity for NAFLD^[17]. A one year, single center nested case controlled study analyzed 928 IBD patients who had any abdominal imaging and found 7.2% had NAFLD^[13]. All included patients did not have clinically significant alcohol consumption to minimize confounding appearance of hepatic steatosis on imaging. Mean age, age at diagnosis, body mass index (BMI) and prevalence of MS were greater in NAFLD patients. Risk factors for NAFLD in IBD were small bowel surgery (OR = 3.7, 95%CI: 1.5-9.3, $P = 0.005$), hypertension (OR = 3.5, 95%CI: 1.5-8.1, $P = 0.004$) obesity (OR = 2.1, 95%CI: 1.05-4, $P = 0.035$) and steroid use at imaging (OR = 3.7, 95%CI: 1.5-9.3, $P = 0.005$). Confounding factors such as nutrition and lifestyle factors were not accounted for in this study. In a large, single-center study of 511 IBD patients, liver steatosis was found in 40% of patients ($P < 0.001$ vs healthy controls)^[14]. In this study, patients with underlying MS and obesity (BMI > 30) were excluded however assessment of nutritional status and physical activity among the cohorts were again not available. Other studies have found 13%-16% rate of hepatic echobright patterns in IBD^[18,19]. Several studies have used liver enzymes derangements to detect NAFLD in IBD, which have poor predictive value to exclude NAFLD^[20]. A one-year prospective analysis of 200 UC patients found 40% with abnormal liver enzymes, with liver biopsy revealing NAFLD in 11.2% of these patients^[21]. A five-year prospective study of IBD (401 UC, 385 CD) showed 15.3% had abnormal liver enzymes^[12]. Ultrasonography of these patients revealed 40.8% had NAFLD, representing 6.2% of all patients. These two studies are also limited by lack of evaluation on relevant confounding factors.

A study from our group using the validated hepatic steatosis index (HSI) longitudinally followed 321 IBD patients over 7 years (217 CD, 104 UC)^[22]. HSI, defined as: $8 \times \text{AST/ALT} + \text{BMI} (+2, \text{ if female}; +2, \text{ if diabetes})$, was applied to diagnose hepatic steatosis if

Table 1 Prevalence of non-alcoholic fatty liver disease and fibrosis in inflammatory bowel disease reported by major studies since 1990

Ref.	Diagnostic method	No. of patients	Mean age	Gender (male)	IBD type	Mean BMI	NAFLD prevalence	Fibrosis
Gisbert <i>et al</i> ^[12]	Ultrasound	786	44		49% (CD) 51% (UC)		40.8%	-
Sourianarayanan <i>et al</i> ^[13]	Ultrasound/CT/MRI	928	44 (NAFLD) 42 (Non-NAFLD)	41%	53% (CD) 47% (UC)	30.4 (NAFLD) 27 (Non-NAFLD)	8.2%	-
Bargiggia <i>et al</i> ^[14]	Ultrasound	511	38 (CD) 39 (UC)	-	61% (CD) 39% (UC)	21 (CD) 21.6 (UC)	39.5% (CD) 35.5% (UC)	-
de Fazio <i>et al</i> ^[18]	Ultrasound	74	35 (CD) 39 (UC)	55%	32% (CD) 68% (UC)		12.0% (CD) 16.6% (UC)	-
Riegler <i>et al</i> ^[19]	Ultrasound	484	38 (CD) 41 (UC)	57%	35% (CD) 65% (UC)		8.9% (CD) 13.6% (UC)	-
Yamamoto-Furusho <i>et al</i> ^[21]	Ultrasound	200	31	53%	UC		11.2	-
Bessissow <i>et al</i> ^[22]	Hepatic steatosis index/Fibrosis-4 score	321	33.7	47%	68% (CD) 32% (UC)	22.2	33.6% (Incidence)	7.4%

NAFLD: Non-alcoholic fatty liver disease; IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis; CT: Computed tomography; MRI: Magnetic resonance imaging.

the score is ≥ 36 . We found the incidence of NAFLD was 33.6% or 9.1/100 patient-years (PY), compared with 0.029 to 3.1/100 PY in the general population. Of those with NAFLD, 7.4% developed advanced liver fibrosis (Fibrosis-4 score > 3.25). The average BMI was 22.2, similar between those who did not develop NAFLD; although we did not capture other nutritional and lifestyle data. NAFLD development was predicted by active disease (HR = 1.58, 95%CI: 1.07-2.33), longer disease duration (HR = 1.12, 95%CI: 1.03-1.23) and prior IBD-related surgery (HR = 1.34, 95%CI: 1.04-1.74). Anti-tumor necrosis factor alpha (Anti-TNF α) therapy trended toward predisposing to NAFLD (HR = 1.69, 95%CI: 0.99-2.9, $P = 0.056$). There was no association between the incident of NAFLD and steroids use. However, steroid use was defined as use at any point prior to a NAFLD diagnosis, which may not appropriately characterize those with repeated or prolonged steroid use.

PATHOGENESIS

Although the pathogenesis for IBD and NAFLD are both poorly understood, these disorders are likely to have arisen from complex interaction of polygenic predisposition with multiple environmental factors. For NAFLD, it is postulated that hepatic steatosis may have developed from insulin resistance and the associated metabolic disturbances leading to fatty infiltration in the liver^[23]. Oxidative damage, immune activation, dysregulated cytokine and apoptosis pathways, are among other processes, further contribute to hepatic insult and fibrogenesis leading to NASH; the so called multi-hit hypothesis. IBD is characterised by dysregulated immune activation through host microbiota dysbiosis and environmental triggers in a genetically predisposed individual^[24]. More than 200 genetic polymorphisms have been

linked to the development of IBD. Similarly several single nucleotide polymorphisms have been found through genome wide association studies that may contribute to the development of NAFLD. There does not however appear to be any definite overlap of genetic predisposition in these two populations, albeit this has not been directly evaluated. Other factors, such as MS, microbial dysbiosis, immune activation, and medications on the other hand may exert more influence in the coexistence of these two disorder and these topics will be discussed in the following sections.

MS

An overlap of the metabolic risk factors for type 2 diabetes and for atherosclerotic cardiovascular disease, such as abdominal obesity, hyperglycemia, dyslipidemia and hypertension have led to the concept of the MS. Its cardinal pathophysiology is insulin resistance due to obesity. NAFLD is thought to be the hepatic manifestation of MS. A recent study demonstrated the prevalence of MS in IBD patients was comparable to that of the general population (18.6%)^[25]. Potential confounding factors, including exercise, sleeping, alcohol intake and smoking did not differ significantly between IBD patients with or without MS; nutritional factors were not assessed by the study. In addition, a trend toward a higher prevalence of MS was found in UC (23%) patients compared to CD (7.1%) patient and in male IBD patients (21.1%) compared to female patients (12.9%). Another study found the prevalence of MS was 10.3% under 45 years of age and 55% over 45 years of age^[26]. Furthermore, they found that it was more prevalent in patients with UC (29.5%) than in patients with CD (17.7%). This study however did not account for potential confounding lifestyle characteristics. A North American study found that the prevalence of MS was lower among their IBD patients both with and without NAFLD compared

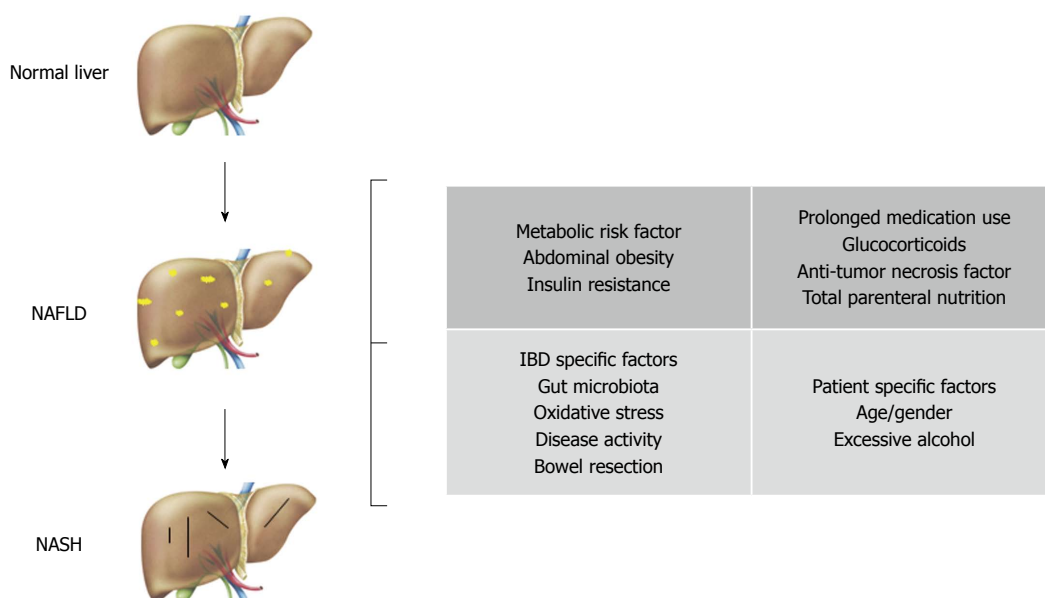


Figure 1 Potential pathogenic factors contributing to the coexistence of non-alcoholic fatty liver disease and inflammatory bowel disease. NAFLD: Non-alcoholic fatty liver disease; IBD: Inflammatory bowel disease.

Table 2 Reported risk factors of non-alcoholic fatty liver disease in inflammatory bowel disease patients

Risk factors	OR/HR (95%CI)	P value
Small bowel surgery ^[13]	OR = 3.7 (1.5-9.3)	0.005
Hypertension ^[13]	OR = 3.5 (1.5-8.1)	0.004
Obesity ^[13]	OR = 2.1 (1.05-4.0)	0.035
Steroid use ^[13]	OR = 3.7 (1.5-9.3)	0.005
Active disease ^[22]	HR = 1.58 (1.07-2.33)	0.020
Duration of IBD ^[22]	HR = 1.12 (1.03-1.23)	0.010
Prior IBD surgery ^[22]	HR = 1.34 (1.04-1.74)	0.020
Anti-TNF α use ^[22]	HR = 1.69 (0.99-2.90)	0.056 (Trend to significance)

IBD: Inflammatory bowel disease; TNF α : Tumor necrosis factor α .

to the general United States population^[13]. MS thus may not be the only dominant factor contributing the coexistence of NAFLD in IBD patients. Nevertheless, early identification and intervention of these metabolic factors may reduce the development of complications associated with NAFLD.

IBD disease factors: Inflammation and dysbiosis

NAFLD in IBD patients was predicted by disease-specific factors in the aforementioned studies which included disease activity and duration, along with prior IBD related or small bowel surgery, steroid and possibly anti-TNF α use. The etiology of IBD and factors provoking exacerbation are still partially understood. Intestinal microbiota have emerged as a key player in the pathogenesis of IBD. Alteration of gut microbiota has been associated with disease activity^[27]. On the same line, NAFLD is associated with increased intestine permeability, and this abnormality is related to the increased prevalence of

small bowel bacterial overgrowth in these patients. As such, alteration of gut microbiota may act as a pathogenic link between IBD and NAFLD. This makes one suspect that an active inflammatory process could drive fatty infiltration of the liver. Similar association have been made between psoriasis and NAFLD^[28]. Duration of IBD was another independent predictor of development of NAFLD in the aforementioned Bessissow *et al.*^[22] study. Longer disease duration exposes patients to multiple risk factors for NAFLD, including chronic relapsing inflammation, alteration of gut microbiota and hepatotoxic drugs. In particular, oxidative stress from reactive oxygen species may also be the common pathogenic factor contributing the consistence of NAFLD and IBD. Along the same lines, prior surgery was also independently associated with incident NAFLD. This is most likely a surrogate marker of the severity of the disease with a more active inflammatory condition. Those patients will also tend to be exposed to hepatotoxic medications repeatedly. NASH development following extensive small bowel resection in non-IBD patients has also been previously described and may be related to nutritional deficiencies akin to those patients with bariatric bypass procedures^[29]. Table 2 summarises the reported risk factors for NAFLD in IBD and Figure 1 depicts the hypothesized pathogenic factors of NAFLD in IBD.

NAFLD and the interactions with IBD therapeutic agents

Glucocorticoids: Glucocorticoid analogues (GC) are commonly used as induction agents for the management of IBD and a subset of patients with poorly controlled disease may have repeated or prolonged exposure. They have profound metabolic effects on carbohydrate and lipid metabolism which

may result in the development of MS and potentially NAFLD. *In vitro* studies have demonstrated that GC may induce lipogenesis and steatosis in hepatocytes *via* several mechanisms including up-regulation of fatty acid synthase and acetyl-CoA carboxylases 1 and 2^[30]. GC and high fat diet in rodent models also can synergistically exacerbate the development of NAFLD and hepatic fibrosis^[31]. However evidence linking GC and NAFLD in human studies are less direct. No prospective clinical study has shown GC use as an independent risk factor for NAFLD. Only 20% of patients with Cushing's syndrome, associated with GC use, have radiological evidence of NAFLD^[32]. Similarly plasma cortisol concentrations do not differ significantly in NAFLD or obese patients as compared to controls^[33]. The retrospective study by Sourianarayanan *et al.*^[13] found steroid use at the time of US imaging was an independent risk factor for NAFLD (OR = 3.7, 95%CI: 1.5-9.3) in the IBD population, however this was not consistently found in other observational studies. Even though no clear guidelines have been established, corticosteroid should be cautiously used in patients with existing metabolic risk factors.

Methotrexate: Methotrexate (MTX) is a folate antagonist which competitively inhibits dihydrofolate reductase and interferes with purine and pyrimidine synthesis, resulting in anti-inflammatory and other effects. It can be used as an induction and maintenance monotherapy for the treatment of IBD, or as combination therapy with anti-TNF α agents^[34]. 15%-50% of patients on methotrexate may develop changes in liver enzymes, although most are self-limiting and the underlying mechanism is presumed relating to oxidative stress^[35]. A retrospective analysis has reported around 24% of IBD patients on MTX have liver enzyme elevations. Significant hepatic fibrosis or cirrhosis, however are uncommon, accounting only for 5% of patients on long term low dose MTX. Association between MTX and NAFLD is less definitive. MTX use has not been shown to result in NAFLD in IBD patients. There is one report in rheumatoid arthritis patients, where average weekly dose of 13.1 mg MTX was shown to be an independent predictor of NAFLD on multivariate analysis^[36]. Despite the lack of associations, there are rodent studies showing increased susceptibility to MTX induced hepatic toxicity in established NAFLD; therefore it may not be entirely appropriate in patients with NAFLD^[37].

Anti-TNF α : TNF α and its participation in pro-inflammatory pathways may play an important role in the development of hepatic inflammation and NASH in NAFLD patients. Significantly elevated serum TNF level as well as messenger RNA expression in hepatocytes have been demonstrated in NASH patients compared to healthy controls^[38,39]. Anti-TNF α agents are widely used in various inflammatory diseases and are by far the

most effective induction and maintenance agents for IBD. It has been postulated that anti-TNF α may protect against NASH. Infliximab has been shown to reduce steatosis and increase insulin signal transduction in rodents on high fat diet^[40]. Furthermore infliximab also reduced hepatic inflammation, necrosis and fibrosis in NASH rodents induced by methionine and choline deficient diet^[41]. Similar effects were also shown with the use of adalimumab^[42]. Finally pentoxifylline, a nonselective phosphodiesterase inhibitor that reduced TNF production, has also been reported to induce biochemical liver enzymes improvement in NASH patients^[43]. On the other hand there are case series reporting the development of biopsy-proven NAFLD in patients receiving anti-TNF α despite no changes in metabolic profiles from improved disease control and enhanced nutrition. In one of the aforementioned NAFLD prevalence studies, there was a trend to significance with the use of anti-TNF α as a risk factor, whereas others did not show any statistically significant associations and one study showed that it may have a protective effect. No clear conclusions thus could be reached on the effect of anti-TNF α in NAFLD/NASH due to the conflicting evidence.

Other common IBD therapeutic agents: Thiopurine analogues, azathioprine and 6-mercaptopurine, remain a corner stone therapy for the maintenance of remission in IBD. They however can be associated with liver function derangement, cholestatic and hepatocellular hepatitis, in addition to veno-occlusive disease, peliosis hepatis and nodular regenerative hyperplasia^[44]. There are no clear evidence linking NAFLD to these agents, nor are there any data suggesting higher risk of thiopurine liver injury in patients with existing NAFLD. Similarly, multiple other therapeutic agents using monoclonal antibodies targeting various inflammatory pathways have been recently approved or being developed for use in IBD, such as vedolizumab and ustekinumab. Currently there are not enough published data to comment on their interactions with NAFLD.

Parenteral nutrition

A small proportion of IBD patients may develop intestinal failure secondary to extensive surgical resection or refractory disease thus requiring parenteral nutrition (PN). Hepatic steatosis is a known common complication and can occur as early as 5 d post PN commencement^[45]. Progressive inflammatory response and fibrosis may also ensue with prolonged exposure. These events may be promoted through excessive caloric and carbohydrate administration. In addition, deficiencies of amino acids such as carnitine and choline as well as essentially fatty acids are also implicated. There are limited evidence suggesting the use of lipid emulsions and optimization of caloric content may help to minimize these complications^[46].

CLINICAL IMPLICATIONS

Screening

According to the American association for the study of liver disease guideline, universal screening in asymptomatic general or high risk populations is not currently recommended due to uncertainties with diagnostic tests, cost-effectiveness and long term benefits^[47].

US is commonly used for the screening and evaluation in patients suspected of NAFLD. Several non-invasive serum biomarker scores, such as the NAFLD liver fat score and fatty liver index have been validated for the assessment of hepatic steatosis^[48]. Cytokeratin 18, another serum test, has a sensitivity of 78%, specificity of 87%, and an area under the receiver operating curve of 0.82 (95%CI: 0.78-0.88) for diagnosing steatohepatitis. Similarly, presence of fibrosis may also be detected with the use of markers including the fibrosis 4 calculator, NAFLD fibrosis score and the elevated fibrosis tests.

There has also been some promisingly development of alternative imaging methods for the detection of liver fibrosis; the most studied being transient elastography (TE) which may assess the presence of advanced fibrosis. The adjunct use of controlled attenuation parameter function of the TE has also been used to diagnosis hepatic steatosis; this however has not been robustly validated in IBD.

No specific guidelines for the assessment of NAFLD in the IBD population have been established. Evaluation may be helpful in IBD patients with high risks or those with imaging features of hepatic steatosis; although the optimal approach and benefits are yet to be studied.

Treatment

The current focus of NAFLD therapy in general is dietary and lifestyle modifications with the aim of weight reduction but no treatment has been assessed in the IBD population specifically. Weight loss > 7% has been associated with biochemical and histological improvement in patients with NASH^[49]. Prevention or reversal of hepatic fibrosis ultimately should lead to reduction of NAFLD related complications. This approach may not be entirely suitable for some IBD patients with existing nutritional deficits in the setting of poorly controlled disease activity. No pharmacological agents have received formal regulatory approval as NASH therapy. Pioglitazone, a peroxisome proliferator-activated receptor agonist, vitamin E and synthetic farnesoid X receptor agonist, obeticholic acid, have all been shown to improve histological markers in NASH^[50,51]. Several anti-inflammatory and anti-fibrosis agents are also being actively investigated. Bariatric surgery also could lead to improved NASH status in morbidly obese patients^[52]. Once again NAFLD treatments have not been specifically studied in the IBD population and the management of these

patients should be individualized and guided by existing protocols for non-IBD patients. The use of IBD treatment agents in the setting of NAFLD has been discussed in the previous paragraphs.

CONCLUSION

The co-existence of NAFLD in IBD is becoming increasingly recognized. This is partly related to an increase in MS as well as complex IBD disease associated factors. Current literature on this matter has left many issues unanswered. Long term outcomes and prognosis for co-existent patients must be characterised. The true impact of IBD therapies on co-existing NAFLD also needs to be further assessed. Furthermore, guidance on the appropriate screening tool and strategies for the management of co-existent disease in IBD patients is lacking. Clarification of these issues may enhance early intervention and improve patient outcomes.

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Inflammatory bowel disease and airway diseases

Maria Vutcovici, Paul Brassard, Alain Bitton

Maria Vutcovici, Alain Bitton, Division of Gastroenterology, McGill University Health Centre, Montreal General Hospital, Montreal, Québec H3G 1A4, Canada

Paul Brassard, Division of Clinical Epidemiology, McGill University Health Centre, Montreal, Québec H3A 1A1, Canada

Paul Brassard, Alain Bitton, Department of Medicine, McGill University, Montreal, Québec H3G 1Y6, Canada

Author contributions: Vutcovici M, Brassard P and Bitton A contributed equally to the conception and design of the manuscript; Vutcovici M conducted the literature review and drafted the manuscript; Bitton A and Brassard P contributed to the critical revision and editing; all authors approved the final version.

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Correspondence to: Maria Vutcovici, MD, MSc, Division of Gastroenterology, McGill University Health Centre, Montreal General Hospital, 1650 Cedar Avenue D16-125, Montreal, Québec H3G 1A4, Canada. maria.vutcovici@mail.mcgill.ca
Telephone: +514-340-8222-3678
Fax: +514-843-2891

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Abstract

Airway diseases are the most commonly described lung manifestations of inflammatory bowel disease (IBD). However, the similarities in disease pathogenesis and the sharing of important environmental risk factors and genetic susceptibility suggest that there is a complex interplay between IBD and airway diseases. Recent evidence of IBD occurrence among patients with airway diseases and the higher than estimated prevalence of subclinical airway injuries among IBD patients support the hypothesis of a two-way association. Future research efforts should be directed toward further exploration of this association, as airway diseases are highly prevalent conditions with a substantial public health impact.

Key words: Inflammatory bowel disease; Ulcerative colitis; Asthma; Chronic obstructive pulmonary disease; Crohn's disease

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Core tip: Recent evidence of inflammatory bowel disease (IBD) occurrence among patients with airway diseases and the higher than estimated prevalence of subclinical airway injuries among IBD patients support the hypothesis of a two-way association between these conditions. Future research efforts should be directed toward further exploration of this association, as airway diseases are highly prevalent conditions with a substantial public health impact.

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INTRODUCTION

An association between inflammatory bowel disease (IBD) and airway diseases has long been described in the literature. The majority of studies have addressed the topic from the perspective of airway diseases as an extraintestinal manifestation of IBD^[1-7]. However, there is growing evidence regarding an increased risk of IBD occurrence among patients with airway diseases such as asthma^[8-11], chronic obstructive pulmonary disease (COPD)^[12,13] and bronchiectasis^[13]. There are several similarities between these conditions, ranging from the multifactorial complex etiology to the chronic remitting-relapsing disease course and the presence of low-grade systemic inflammation. It is, therefore, likely that the complex relationship between IBD and airway diseases is not merely unidirectional, and the new evidence from population-based studies supports this hypothesis.

In this paper we review the similarities between airway diseases and IBD and address the epidemiological evidence for the association, focusing on IBD occurrence in patients with airway diseases.

SIMILARITIES IN PATHOGENESIS AND RISK FACTORS

Genetic factors

Genome-wide association studies have shown an overlap of regions of genetic linkage for asthma, IBD, and other autoimmune disorders^[14]. Several gene loci, such as DENND1B, SMAD3 and SLC22A4/5 (5q31/IBD5), were found to be associated with both asthma and Crohn's disease (CD), while the ORMDL3 gene variants present in CD and ulcerative colitis (UC) were also found in childhood-onset asthma^[15]. The association between NOD2 gene polymorphism and the development of both CD^[16] and COPD^[17] supports the hypothesis of a shared genetic susceptibility. NOD2 proteins recognize peptidoglycan components of the bacterial wall, contributing thus to bacterial recognition and the activation of immune defense pathways^[17].

Embryologic origin, anatomical structure and function

The epithelia of the intestine and airways derive from the same embryological structure, the foregut region of the endoderm^[18]. Their anatomical structure is, therefore, very similar, with a columnar type epithelium, goblet cells and mucous glands^[4,19,20]. The lymphoid tissue in the submucosal layer is composed of antigen-presenting cells and lymphocytes capable of releasing pro-inflammatory cytokines^[21], and plays an important role in both innate and adaptive immune defense as part of the barrier organ function of the

respiratory and gastrointestinal tracts^[4].

Pathogenesis

Several similarities in the underlying pathological mechanisms have been described and may explain the association between IBD and airway diseases^[1,2,22-24]. Dysbiosis and an inappropriate immune response to intestinal microbiota are considered key components of the pathophysiological process in IBD^[25]. Similarly, an immune response to lung microbiota seems to occur in airway diseases such as bronchiectasis^[26]. A dysregulation of protease activity is present in both IBD^[27] and COPD^[28], and is associated with the breakdown of connective tissue components and the ensuing remodeling process^[1,29]. Alterations in immune cell homing function^[1,22] may explain the low grade chronic systemic inflammation that is present in IBD^[30], COPD^[31], asthma^[32-34] and bronchiectasis^[35-38]. The hygiene hypothesis, proposing that a lack of exposure to microorganisms during childhood contributes to abnormal immune reactions later in life, may also constitute a common factor linking asthma, IBD and a variety of other conditions^[39].

Environmental factors

Tobacco smoking is an important risk factor associated with the development of both airway diseases^[40,41] and CD^[42]. In asthmatic patients, smoking is associated with a decline in lung function^[43] and increased morbidity rates^[41,44]. A relative resistance to corticosteroid therapy was reported in smoking asthmatic and COPD patients^[41], and the hospitalizations and mortality rates were higher than in non-smoking asthma and COPD controls^[40,45]. Smoking has been associated with a poor response to treatment and a more severe disease course in CD patients^[42,46,47]. In contrast, ulcerative colitis seems to be a disease of non-smokers or former smokers^[48], and a more benign disease course was observed in UC smokers compared to non-smokers^[49]. This suggests that the association between UC and airway diseases goes beyond the confounding effect of smoking. Several potential underlying mechanisms that may explain the effect of cigarette smoke in IBD have been advanced, including alterations in cellular and humoral immunity, alterations in mucosal blood flow, gut permeability and motility, as well as a pro-thrombotic and a reduced anti-oxidant effect^[42], but the relationship with the dichotomous impact on CD and UC is still unclear.

Air pollution is another environmental risk factor associated with both airway diseases and IBD. Air pollutants such as particulate matter, ozone or nitrous oxides were associated with an increase in number of hospitalizations for asthma^[50], COPD^[51-53] and IBD^[54], and with an increased risk of mortality in COPD patients^[55]. The gastrointestinal tract is exposed to air pollutants through contaminated food and water^[56]. There appears to be a dose-response association

between long term exposure to air pollutants, such as nitrous oxides and particulate matter, and the risk of early onset CD^[57]. Exposure to sulfur oxides was associated with an increased risk of early onset UC, but no dose-response effect could be demonstrated^[57]. Gastrointestinal injury may be the result of alterations in gut microbiota induced by exposure to air pollutants, as demonstrated in animal models^[58], of an increased intestinal permeability, or of a pro-inflammatory effect^[56].

Vitamin D is an environmental factor with a pleiotropic role in immune regulation^[59], from inhibiting cytokine production to enhancing innate immunity by facilitating the transcription of peptides with antimicrobial effects^[60]. Low serum levels of Vitamin D in asthmatic patients were associated with impairments in lung function, a poor response to corticosteroid therapy and an increased airway hyper-reactivity^[61]. In children with mild to moderate asthma, vitamin D deficiency is relatively common and associated with an increased risk of severe exacerbations^[62]. Vitamin D deficiency in IBD patients may be a consequence of malabsorption, low dietary intake or reduced bioavailability^[63], but the suboptimal serum levels observed in newly diagnosed patients^[64] suggest that the deficiency may also be associated with IBD development^[59,63]. A randomized control trial of Vitamin D supplementation in CD patients showed a reduced number of relapses compared to the placebo group^[65]. Further studies are needed to confirm this effect.

AIRWAY DISEASE IN IBD

Airway diseases were first described in IBD patients four decades ago in the case series of Kraft *et al.*^[66]. There is an extensive literature documenting airway diseases as an extraintestinal manifestation of IBD. Approximately 6%-47% of patients develop at least one extraintestinal manifestation^[67-69] during the course of IBD, but the true prevalence of lung involvement is unknown due to the presence of subclinical pulmonary injury. It is estimated that 40%-60% of IBD patients have some degree of subclinical lung involvement evidenced through alterations in pulmonary function tests and high resolution tomographic imaging (HRCT)^[70-73].

The most frequently observed alterations in pulmonary function tests are decreases in forced expiratory volume in 1 s (FEV₁)^[72,73] and FEV₁/forced vital capacity (FVC) ratio, in forced expiratory flow 25%-75%^[72,73] as well as in the transfer coefficient for carbon monoxide (DLCO)^[73]. The severity of the observed alterations in pulmonary function tests was found to be in correlation with the endoscopic and clinical activity in UC patients^[73,74] and independent of the effect of smoking^[73].

HRCT imaging techniques allow the detection of lung involvement in IBD patients without overt respiratory symptoms. The most common findings are an enlarged bronchial internal diameter, peribronchial wall thickening, air trapping or the identification of

airways in the extreme lung periphery^[6,75]. The imaging appearance of small airway involvement in UC patients, with the "tree in bud" aspect and cellular bronchiolitis, was described as indistinguishable from the imaging findings in patients with rheumatoid arthritis or in transplant recipients, indicating thus an immunological mechanism of small airway injury^[75].

In IBD patients with respiratory symptoms, airway diseases are the most commonly reported respiratory condition^[4,11,73,76]. Bronchiectasis was found to occur in 22% of symptomatic cases^[4,5], followed by chronic bronchitis in 20% of cases^[5] and suppurative airway disease without bronchiectasis^[4]. Furthermore, evidence from population-based epidemiologic studies indicates an association with asthma, bronchitis and COPD. A large matched-cohort study involving more than 8000 IBD patients found asthma to be the second most common comorbidity after arthritis in both CD and UC^[11]. The prevalence of bronchitis was also significantly increased in IBD patients compared to healthy controls^[11]. Studies of survival and cause of death reported a significant increase in mortality due to COPD among IBD patients^[77,78].

Lung involvement in IBD can also result from the effect of IBD-specific medications. The most commonly reported associations were with interstitial lung diseases, such as interstitial pneumonitis (for Mesalamine, thiopurines and biologics) or diffuse interstitial lung disease (for Methotrexate)^[3], or with diseases affecting the lung parenchyma, such as eosinophilic pneumonia (for Mesalamine and biologics)^[79,80], but not with airway diseases.

IBD OCCURRENCE IN AIRWAY DISEASES

Despite the substantial evidence of an IBD-airway disease association and of the complex interplay between the two groups of conditions, an interest toward the possibility of IBD occurrence in patients with pre-existing airway diseases has only recently emerged. In the last decade, a handful of population-based studies have addressed the risk of developing IBD, its incidence or prevalence in patients with asthma, bronchitis, bronchiectasis and COPD (Table 1).

Four studies have reported an increased prevalence of IBD in patients with airway diseases. In the study of Bernstein *et al.*^[11], the prevalence of CD and UC among patients with asthma and bronchitis was significantly increased compared to the prevalence in the general population of Manitoba, Canada. A four-fold increase in IBD prevalence was observed in a cohort of patients with airway diseases in the United Kingdom. The prevalence of UC was significantly increased in all types of airway disease investigated; CD prevalence was increased in patients with COPD and bronchiectasis^[13]. A study of COPD patients and their first degree relatives identified from the Swedish Multigeneration Register showed an increased prevalence of both CD and UC among the patients

Table 1 Population-based studies of inflammatory bowel disease occurrence in patients with airway diseases

Ref.	Country	Airway disease	Cohort size	Results		
				Crohn's disease	Ulcerative colitis	IBD
Bernstein <i>et al</i> ^[11]	Canada	Asthma	12397	PRR = 1.38 (95%CI: 1.23-1.53)	PRR = 1.56 (95%CI: 1.4-1.74)	
		Bronchitis	3092	PRR = 1.72 (95%CI: 1.15-2.58)	PRR = 1.92 (95%CI: 1.35-2.73)	
Ekbom <i>et al</i> ^[12]	Sweden	COPD	180239	HR = 2.72 (95%CI: 2.33-3.18)	HR = 1.83 (95%CI: 1.61-2.09)	
Raj <i>et al</i> ^[13]	United Kingdom	COPD	588	OR = 5.26 (95%CI: 1.71-16.19)	OR = 3.57 (95%CI: 1.3-9.38)	OR = 3.87 (95%CI: 1.19-12.62)
		Bronchiectasis	215	OR = 7.21 (95%CI: 1.62-32.2)	OR = 7.88 (95%CI: 2.71-22.91)	OR = 8.38 (95%CI: 2.43-28.89)
		Asthma	893	OR = 1.74 (95%CI: 0.39-7.65)	OR = 2.81 (95%CI: 1.15-6.90)	OR = 2.54 (95%CI: 0.78-8.26)
		Airway disease, total	2192	OR = 5.96 (95%CI: 1.94-18.31)	OR = 4.21 (95%CI: 1.71-10.41)	OR = 4.26 (95%CI: 1.48-11.71)
Virta <i>et al</i> ^[9]	Finland	Asthma	185	OR = 2.33 (95%CI: 1.41-3.86)	OR = 1.11 (95%CI: 0.68-1.80)	
Hemminki <i>et al</i> ^[8]	Sweden	Asthma	148295	SIR = 1.64 (95%CI: 1.42-1.87)	SIR = 1.54 (95%CI: 1.36-1.73)	
Brassard <i>et al</i> ^[10]	Canada	COPD	143904	IRR = 1.55 (95%CI: 1.49-1.62)	IRR = 1.30 (95%CI: 1.24-1.37)	
		Asthma	136178	IRR = 1.27 (95%CI: 1.22-1.31)	IRR = 0.99 (95%CI: 0.94-1.04)	

IBD: Inflammatory bowel disease; PRR: Prevalence rate ratio; COPD: Chronic obstructive pulmonary disease; SIR: Standardized incidence ratio; IRR: Incidence rate ratio; CI: Confidence interval; HR: Hazard ratio; OR: Odds ratio.

and their siblings compared to IBD prevalence in controls^[12]. Younger age at COPD diagnosis was found to be associated with a higher prevalence of UC. A case-control study of Finnish children with IBD showed that the risk of developing CD was significantly higher in children with asthma and with both asthma and cow milk allergy than in healthy controls^[9].

Unfortunately, prevalence studies are informative only from the point of view of disease coexistence, and cannot provide an insight into the temporal sequence of developing these conditions. Two population-based studies assessed IBD occurrence during the course of airway diseases, one in asthmatic patients only and the other in asthma and COPD.

A study of Swedish inpatients discharged with a diagnosis of asthma showed an increased incidence of CD and UC hospitalizations during the follow up period^[8]. To ensure incident IBD cases were captured, all subjects in which the diagnosis of CD and UC preceded that of asthma were excluded from the analyses. The standardized incidence rates for CD were significantly increased in all age groups, while the incidence increase of UC was only significant in subjects diagnosed with asthma after the age of 20 years.

More recently, a large retrospective cohort study of Québec patients with asthma and COPD showed a significantly increased incidence of both CD and UC in COPD patients and an increased incidence of CD in asthmatic patients compared to the IBD incidence in the general population^[10]. Similar to the results of Hemminki *et al*^[8], in asthmatic patients the incidence of CD was significantly increased in all age groups; the incidence of UC, although not significantly increased when all age groups were considered, was significantly increased in patients diagnosed with asthma after the age of 10 years. In COPD patients, the incidences of both CD and UC were significantly increased compared to the general population for all age groups. A follow

up study of the same COPD cohort revealed that new onset IBD was associated with an increased risk of all-cause mortality as well as from respiratory and digestive causes^[81].

FUTURE DIRECTIONS FOR RESEARCH

The existing evidence of IBD occurrence in patients with airway diseases is supported by population based studies. In clinical settings, the true prevalence of IBD or of digestive symptoms indicative of IBD is still unknown. If clinical studies confirm the association, it would be of importance to assess whether exacerbations in airway diseases are impacted by IBD disease activity.

Inversely, in IBD patients with lung manifestations, further assessment of prevalence of subclinical airway injuries is warranted, as recent evidence suggests it is much higher than previously expected. Such an assessment could shed more light into the temporal sequence of IBD-airway disease development.

CONCLUSION

There is a complex inter-relation between IBD and airway diseases and new evidence suggests that not only can airway diseases occur as an extraintestinal manifestation of IBD, but that IBD, in turn, can have a high occurrence in patients with airway diseases. This occurrence seems to also impact on key outcomes, such as mortality in COPD patients. While the importance of airway involvement as extraintestinal manifestation of IBD is amplified by the evidence of an increased prevalence of subclinical airway injuries, the impact of IBD occurrence in patients with airway diseases raises a substantially greater public health concern due to the worldwide high prevalence of airway diseases. Early detection of IBD may improve the treatment management and prognosis of airway disease patients.

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Spontaneous fungal peritonitis: Epidemiology, current evidence and future prospective

Marco Fiore, Sebastiano Leone

Marco Fiore, Department of Anesthesiological, Surgical and Emergency Sciences, Second University of Naples, 80138 Naples, Italy

Sebastiano Leone, Division of Infectious Diseases, "San Giuseppe Moscati" Hospital, 83100 Avellino, Italy

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Correspondence to: Sebastiano Leone, MD, Division of Infectious Diseases, "San Giuseppe Moscati" Hospital, Contrada Amoretta, 83100 Avellino, Italy. sebastianoleone@yahoo.it
Telephone: +39-825-203967
Fax: +39-825-203967

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Abstract

Spontaneous bacterial peritonitis is a complication of ascitic patients with end-stage liver disease (ESLD);

spontaneous fungal peritonitis (SFP) is a complication of ESLD less known and described. ESLD is associated to immunodepression and the resulting increased susceptibility to infections. Recent perspectives of the management of the critically ill patient with ESLD do not specify the rate of isolation of fungi in critically ill patients, not even the antifungals used for the prophylaxis, neither optimal treatment. We reviewed, in order to focus the epidemiology, characteristics, and, considering the high mortality rate of SFP, the use of optimal empirical antifungal therapy the current literature.

Key words: Cirrhosis; Critically ill patient; Spontaneous fungal peritonitis; Life-threatening infections; Fungal ascitis; Nosocomial spontaneous peritonitis

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Core tip: Spontaneous bacterial peritonitis (SBP) occurs in patients with end-stage liver disease (ESLD); spontaneous fungal peritonitis (SFP) is a complication of ESLD less known and described. Patients with SFP had a significantly worse prognosis than those with SBP. The incidence accounts from 0% to 13% of patients with ESLD and spontaneous peritonitis. Data are conflicting regarding fungi distribution between nosocomial and non-nosocomial infections. *Candida* spp. are the most frequent fungal infectious agent isolated. Previous SBP antibiotic prophylaxis, hepatorenal syndrome, low ascitic fluid protein (< 1 g/dL), elevated acute physiology and chronic health evaluation II and serum lactate also significantly adversely impact hospital mortality.

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INTRODUCTION

Spontaneous bacterial peritonitis (SBP) occurs in patients with end-stage liver disease (ESLD); however, spontaneous fungal peritonitis (SFP) is a complication of ESLD less known and described. A diagnosis of SFP is based on large numbers of neutrophil granulocytes (> 250 cells/mL) of ascitic fluid and diagnostic investigation to exclude other causes of intra-abdominal infection^[1], whereas we define fungal ascitis as fungal culture positive in ascitic fluid in the presence of ascitic neutrophil counts lower than 250 neutrophils/mL. Hospital-acquired (HA) spontaneous peritonitis (SP), both HA-SBP and HA-SFP, is peritonitis that occurs 48-72 h after hospitalization in the absence of signs of infection at hospital admission.

EPIDEMIOLOGY

Asia

Hwang *et al.*^[1] evaluated ESLD patients with SP between 2000 and 2005 in a Korean tertiary care center: 401 patients with SBP and 15 with SFP (3.6%); eleven of the 15 SFP was polymicrobial (Table 1). SFP was more common in nosocomial SP and in patients with higher Child-Turcotte-Pugh (CTP). The most commonly fungus found was *Candida* spp (8 patients *C. albicans*; 1 patient *C. tropicalis*; 1 patient *C. glabrata*), followed by *Cryptococcus neoformans* (5 patients). More than two-thirds of patients (11 patients, 73.3%) with fungal infection died within the first month after diagnosis of SP. All 10 patients showed no improvement with empirical antimicrobial therapy and died within a month. Of 5 patients who showed improvement with empirical antimicrobial therapy, only one died in the first month for gastrointestinal bleeding; the remaining four patients survived. HA-SBP and community-acquired SBP (CA-SBP) occurred in 151 and 265 patients, respectively. Distribution of fungi between HA-SFP vs CA-SFP was 12 vs 3, respectively. The mean value of CTP score was 12.5 ± 2.0 in the SFP cohort and 11.1 ± 1.7 in the SBP cohort^[1].

In another retrospective study conducted in Korea, between January 1st 2003 and December 1st 2010, ninety-five ESLD patients with SP were included. Among the forty-seven pathogens isolated, one (2.2%) was a *Candida* spp. The patient with *Candida* spp in ascitic fluid had hepatocellular carcinoma and died of liver failure shortly after admission^[2].

Cheong *et al.*^[3] evaluated the clinical difference between SP acquired in the hospital or in the community in patients with ESLD between 2000 and 2007 in a Korean tertiary care center: HA-SBP occurred in 126 and CA-SBP occurred in 110 patients. Distribution of fungi between HA-SFP vs CA-SFP was 2.4% (3 patients) vs 0%.

Li *et al.*^[4] evaluated the drug resistance profile of pathogens isolated by ascitic fluid of 288 Chinese patients with ESLD between 2011 and 2013. Three

hundred and six pathogens were isolated: 207 non-nosocomial and 99 nosocomial infections. Fungi were found in the ascitic fluid of nine patients (2.9%); there was significant difference regarding fungi distribution between nosocomial (7.1%, 7 patients) and non-nosocomial (0.9%, 2 patients) cases ($P = 0.004$).

Jindal *et al.*^[5] recently evaluated the outcome of carbapenem- vs cephalosporin-regimen in Indian cirrhotic patients with SP. A total of 175 patients were enrolled, of these two patients (1.1%) had SFP (1 patient with *Candida* spp and 1 patient with *Aspergillus* spp) and were treated with success.

Europe

Piano and Angeli reviewed microbiological data between 2007 and 2009 of a tertiary care center of northern Italy. Of sixty-nine culture positive SP, two (3%) were SFP. Fluconazole-susceptible *C. albicans* was isolated in the two cases^[6].

In an observational study conducted in 4 university hospitals in north-eastern France, between January 1st 2010 and December 31st 2011, one hundred and ninety ESLD patients had ascites (median age 61.5 years, 58.5% CTP C): 268 ascitic fluid positive culture were obtained. Of these 140 were bacterascites and 57 SBP. Fungi were found in 2.1% of patients with bacterascites and none of SP patients. Bacterascites seems to be considered a serious condition given the mortality rate (close to 20%). The authors concluded that bacterascites is probably a surrogate marker of advanced liver disease^[7].

In order to evaluate the different etiology between of HA- and CA-SBP, ninety-five SP episodes were reviewed from a French Liver Unit. Seventy-eight microorganisms were found (39 isolates in each group) including 1 yeast (*C. albicans*). Distribution of *C. albicans* between HA-SFP vs CA-SFP was 0% vs 2.5% (1 patient)^[8].

Friedrich *et al.*^[9] evaluated the drug resistance profile of pathogens isolated from ascitic fluid of 311 ESLD patients (hospitalized in a German tertiary care center) with their first episode of SP between 2007 and 2013. A total of 138 pathogens were isolated (49 non-nosocomial and 89 nosocomial). Fungal infections, *Candida* spp, were found in 10 patients (7.2%); *C. albicans* (3.6%) is the most frequent fungal infectious agent isolated. Interestingly, there was no significant difference regarding *Candida* spp distribution between nosocomial (9.0%, 8 patients) and non-nosocomial (4.1%, 2 patients) cases ($P = 0.287$).

Reuken *et al.*^[10] reviewed retrospectively 244 positive ascitic fluid culture isolated from ESLD patients between 2000 and 2011 in a German tertiary hospital, of these 90 were documented as monomicrobial SP. Fungal infections, *Candida* spp, were found in 3 patients (3.3%) of the ninety with SP.

Umgelter *et al.*^[11] analyzed prospectively 41 positive ascitic fluid culture isolated from ESLD patients between 2000 and 2011 in a German university medical center.

Table 1 Polymicrobial infections

Ref.	HA-SBP definition	Study design	Data provided by the author	Setting	Patients with polymicrobial infections	Fungal polymicrobial infections
Friedrich <i>et al</i> ^[9] , 2015	PMN > 250 > 48 h of hospitalization	Retrospective cohort	No	University Hospital	24/138	N/A
Li <i>et al</i> ^[4] , 2015	PMN > 250 > 48 h of hospitalization	Retrospective cohort	No	University Hospital	16/306	N/A
Hwang <i>et al</i> ^[1] , 2014	PMN > 250 > 72 h of hospitalization	Retrospective cohort	No	University Hospital	N/A	11/15
Ariza <i>et al</i> ^[12] , 2012	PMN > 250 > 48 h of hospitalization	Retrospective cohort	No	University Hospital	15/261	N/A
Umgelter <i>et al</i> ^[11] , 2009	PMN > 50 > 48 h of hospitalization	Prospective cohort	Yes	University Hospital	4/41	2/2
Bert <i>et al</i> ^[8] , 2003	PMN > 250 > 48 h of hospitalization	Retrospective cohort	No	University Hospital	7/78	N/A

HA: Hospital-acquired; SBP: Spontaneous bacterial peritonitis; PMN: Polymorphonuclear; N/A: Not available.

C. albicans was found in 2 patients (4.8%) both in association with bacterial infections (Table 1). All *C. albicans* were susceptible to fluconazole.

In a retrospective observational study on a cohort of cirrhotic patients with SP conducted in a Spanish teaching hospital, between 2001 and 2009, 261 ascitic fluid culture positive SP were evaluated. The authors excluded from the analysis 15 cultures because polymicrobial, so SFP in this cohort could be underestimated. Distribution of *C. albicans* between HA-SFP vs CA-SFP was 0% vs 0.005% (1 patient)^[12].

Africa

In a prospective study carried out in an Egyptian intensive care unit (ICU) from January to August 2013, 46 patients with ESLD were enrolled. Three patients had a polymorphonuclear (PMN) cell count greater than 250 cells/mL in ascitic fluid, of these 3 patients 1 patient had ascitic and blood culture negative, 2 patients (4.3%) had fungal growth in ascitic fluid: 1 patient had ascitic and blood culture positive for *A. niger* and 1 patient had ascitic culture positive for *C. albicans* and blood culture positive for *C. albicans* and *C. tropicalis*. Three (6.5%) patients had a PMN cell count lower than 250 cells/mL in ascitic fluid, of these 1 had ascitic and blood culture positive for *C. albicans*, 1 had ascitic culture positive for *C. albicans* and blood culture negative, 1 had ascitic culture positive for *A. niger* and blood culture negative. Of these 6 patients only 1 patient who had ascitic and blood culture negative died. Independent risk factors for a fungal infection were found to be previous antibiotic prophylaxis for SBP, hepatorenal syndrome and low protein ascites with total protein concentration of less than 1 g per deciliter. Patients with SFP presented worse prognosis than patients with SBP^[13].

North America/miscellaneous

Karvellas *et al*^[14] conducted a retrospective cohort study involving cirrhotic patients with SBP from 28 hospitals of Canada, United States and Saudi Arabia between 1996 and 2011 presenting with septic shock: a positive culture (blood or ascitic fluid) was found in 86 (68%) of 126 patients enrolled (53 HA-SFP vs 73 CA-SFP), the most common pathogens isolated were *Escherichia coli* (27.3%) followed by *Candida* spp (11.1%): 9 *C. albicans* and 2 *C. glabrata/tropicalis*. No one of these 11 patients survived to hospital discharge. SP-associated septic shock has a poor prognosis (mortality 80%). Appropriate antimicrobial therapy should be given as soon as possible: non-administration corresponds to an increase of 1.86 times hospital mortality per hour. Others hospital mortality risk factors are elevated acute physiology and chronic health evaluation II (APACHE II) and serum lactate.

CURRENT EVIDENCE

Fungi are common saprophytes of the human organism, being ubiquitously on skin and mucous membranes. Antibiotics (used for the prevention of SBP in patients with ascites) acting on the intestinal bacterial flora produce an excessive growth of fungi especially of the intestinal tract^[15] with subsequent "translocation" from the gut lumen across the mucosa into the peritoneal cavity. Immunosuppression and malnutrition, common in ESLD patients, promote this process.

Differences between SBP and SFP: compared with SBP patients, the CTP score seems to be higher in SFP patients^[1]. Patients with SFP had significantly higher mortality than the patients with SBP^[1,13]. Patients who do not respond to empiric antimicrobial therapy

(if this does not cover the fungus) have a very poor prognosis (mortality 100%)^[1]. Data are conflicting regarding fungi distribution between nosocomial and non-nosocomial infections, cases of fungal peritonitis is not clearly more common in HA-^[1,4] than CA-SP^[9]. The number of isolates is so low that any analysis is underpowered so a meta-analysis of observational studies could clarify the fungi distribution between nosocomial and non-nosocomial infections.

C. albicans is the most frequent fungal infectious agent isolated^[1,2,9-11,14] following by *C. neoformans*^[1] and *Aspergillus* spp^[5,13]. Fungal infection is often polymicrobial (73.3%-100% of cases), on the contrary polymicrobial bacterial infections affecting 5.2%-17.4% of cases (Table 1).

Risk factors for hospital mortality in SFP are SBP antibacterial prophylaxis, hepatorenal syndrome, low protein ascites with total protein concentration of less than 1 g per deciliter^[13], elevated APACHE II and serum lactate^[14].

Bremmer *et al*^[16] in an historic cohort study including 25 patients (21 liver transplanted and 4 not liver transplanted), with isolation of *Candida* spp in ascitic fluid, found that *C. albicans* (48%; 12 out of 25) is the commonest pathogen, less frequently *C. glabrata* (20%), *C. parapsilosis* (16%), *C. tropicalis* (12%) and *C. zeylanoides* (4%). In the study, 28-d mortality was significantly higher in patients with elevated Charlson Comorbidity Index, Model for End-Stage Liver Disease (MELD) and APACHE II scores. There was no significant difference regarding 28-d mortality between fungal ascitis and SFP; conversely there was significant difference regarding 28-d mortality between patients who do not underwent liver transplantation (14 out of 21) and patients who underwent liver transplantation (0 out of 4). This study suggests that antifungal therapy used to treat SFP, could be a "bridge" to liver transplantation.

Saludes *et al*^[17] recently reported 7 episodes of *Candida* spp isolation in ascites of cirrhotic patients detected in a Spanish hospital during the past 15 years. *C. albicans* was isolated in 5 patients (71.4%) and *C. glabrata* in 2 patients (28.6%). All patients were CTP C, per year mortality was 100% and 3 patients died in the first 10 d of diagnosis.

Choi *et al*^[18] reviewed the clinical and laboratory features of all cirrhotic patients whose ascites samples were positive for *Candida* spp. A total of 21 cirrhotic patients was identified. Patients were regarded as having peritonitis if they had 1 or more clinical symptom(s) or sign(s) in the absence of any other possible explanation. Ten patients (47.6%) were classified into the spontaneous *Candida*-related peritonitis group, and the remaining 11 patients (52.4%) were classified into asymptomatic fungal ascitis. Mortalities were higher in the spontaneous *Candida* peritonitis group at discharge (50.0% vs 27.3%), 6-mo (90% vs 45.5%) and 1-year (100% vs 54.5%) ($P = 0.007$). Receiver-operating characteristic curve analysis revealed that the cut-off

value of ascitic fluid PMN cell count of 315 cells/mL had the highest diagnostic accuracy with both sensitivity and specificity.

FUTURE PROSPETIVES

The available data suggest that the SFP could affect negatively the prognosis of patients with SP, therefore new diagnostic and therapeutic strategies are required. *Candida* spp. Is associated with a severe outcome when manifested with peritonitis^[18]. In a recent clinical trial, fluconazole was added in patients with HA-SBP with no response to meropenem and daptomycin. In this study, never previously proposed, the authors added empiric antifungal therapy in a therapeutic HA-SBP protocol^[19], although in the latest guidelines no mention is made about the use of antifungals in ESLD patients^[20,21]. Actually start antifungal therapy as soon as possible improves prognosis in patients with invasive candidiasis^[22,23].

Mortality from SFP is increased in case of severe underlying diseases and/or if initial antimicrobial therapy is inappropriate^[14,24-26]. Karvellas *et al*^[14] state that non-administration of an appropriate antimicrobial therapy corresponds to an increase of 1.86 times hospital mortality per hour. Unfortunately, it is not possible to extrapolate from this study the subgroup of SFP, but we can assume that septic shock has a worse outcome.

Area of uncertainty that remains for clinicians is the management of fungal ascitis: studies report no differences in mortality rates among patients with ascitic cell count upper or lower 250 cells/mL^[16], or higher mortality in the SFP group but with a fungal ascitis mortality ranging from 27.3% at discharge to 54.5% after 1 year of discharge^[18], conversely bacterascites show lower mortality rates than SBP^[7].

As we recently proposed, given the low incidence of the SFP, a prophylaxis would be useless^[27]. Treatment should be considered in absence of a positive culture in patients with a higher Charlson Comorbidity Index, MELD and APACHE II scores. Patients with a positive fungal culture of the ascitic fluid independently of PMN count should be treated.

Echinocandins are recommended for patients with HA-SFP or patients with CA-SFP and severe underlying illness given the poor prognosis of inappropriate antimicrobial therapy^[28]. De-escalation to fluconazole is recommended when sensitivity tests are available^[29].

Echinocandins should be considered as empirical or preemptive systemic antifungal therapy for patients with suspected SFP. The de-escalation to fluconazole reduces pharmaceutical costs and emerging of resistant microorganisms^[30].

Micafungin in a different setting of patients with ESLD (liver transplant patients with a MELD score ≥ 20) showed non inferiority to standard antifungal prophylaxis, although renal function showed a better performance in micafungin group^[31]. In conclusion

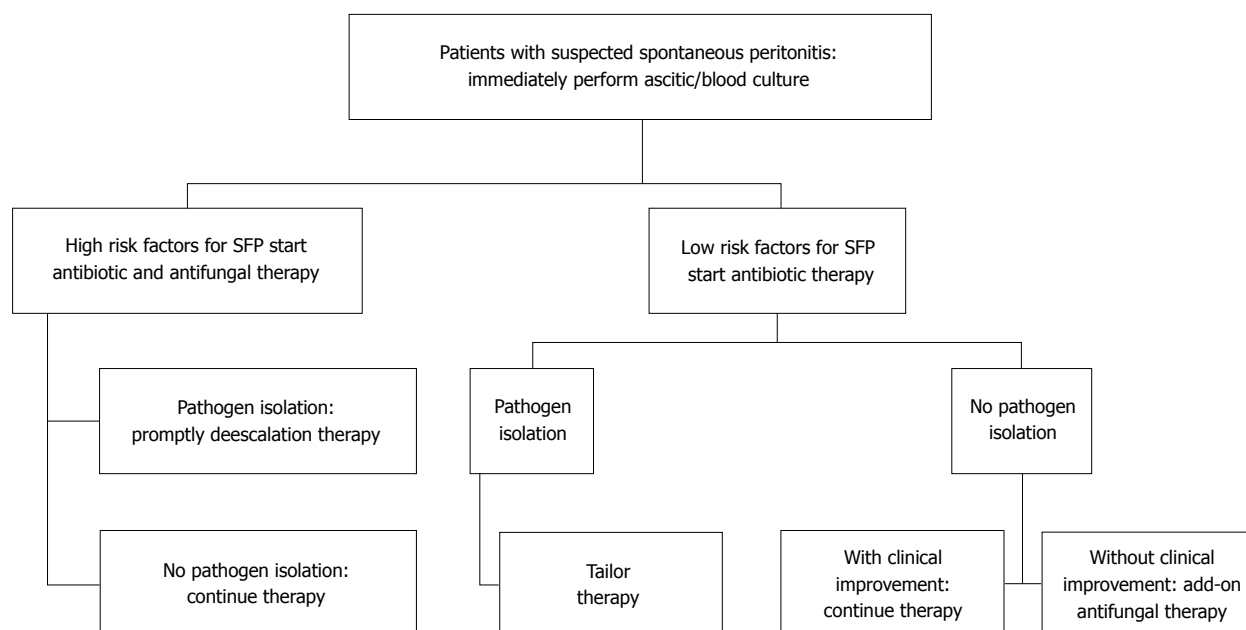


Figure 1 Spontaneous fungal peritonitis management algorithm. Risk factors for fungal diseases^[32]: Surgery, total parenteral nutrition, fungal colonisation, renal replacement therapy, infection and/or sepsis, mechanical ventilation, diabetes, and APACHE II or III score; Add-on: consider adding empiric antifungal therapy. APACHE: Acute physiology and chronic health evaluation.

an algorithm should be proposed for the treatment of patients with suspected SFP (Figure 1).

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Seventh tumor-node-metastasis staging of gastric cancer: Five-year follow-up

Stefano Rausei, Laura Ruspi, Federica Galli, Vincenzo Pappalardo, Giuseppe Di Rocco, Francesco Martignoni, Francesco Frattini, Francesca Rovera, Luigi Boni, Gianlorenzo Dionigi

Stefano Rausei, Laura Ruspi, Federica Galli, Vincenzo Pappalardo, Giuseppe Di Rocco, Francesco Martignoni, Francesco Frattini, Francesca Rovera, Luigi Boni, Gianlorenzo Dionigi, Department of Surgery, University of Insubria, 21100 Varese, Italy

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Correspondence to: Stefano Rausei, MD, PhD, Department of Surgery, University of Insubria, Viale Borri 57, 21100 Varese, Italy. stefano.rausei@asst-settelaghi.it
Telephone: +39-332-278867
Fax: +39-332-260260

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Abstract

Seventh tumor-node-metastasis (TNM) classification for gastric cancer, published in 2010, introduced changes in all of its three parameters with the aim to increase its accuracy in prognostication. The aim of this review is to analyze the efficacy of these changes and their implication in clinical practice. We reviewed relevant Literature concerning staging systems in gastric cancer from 2010 up to March 2016. Adenocarcinoma of the esophago-gastric junction still remains a debated entity, due to its peculiar anatomical and histological situation: further improvement in its staging are required. Concerning distant metastases, positive peritoneal cytology has been adopted as a criterion to define metastatic disease: however, its search in clinical practice is still far from being routinely performed, as staging laparoscopy has not yet reached wide diffusion. Regarding definition of T and N: in the era of multimodal treatment these parameters should more influence both staging and surgery. The changes about T-staging suggested some modifications in clinical practice. Differently, many controversies on lymph node staging are still ongoing, with the proposal of alternative classification systems in order to minimize the extent of lymphadenectomy. The next TNM classification should take into account all of these aspects to improve its accuracy and the comparability of prognosis in patients from both Eastern and Western world.

Key words: Gastric cancer; Staging system; Tumor-node-metastasis; Prognostic factors; Clinical practice

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Core tip: After five years since latest tumor-node-metastasis (TNM) classification for gastric cancer staging has been published, we reviewed Literature concerning its accuracy in prognostication and the impact on clinical practice of the statements introduced in 2010. While waiting for the next UICC/AJCC TNM classification for gastric cancer, open issues and new proposals are also critically discussed.

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INTRODUCTION

Gastric cancer is the fifth most common malignancy and the third leading cause of cancer-related death^[1]. In the era of stage adapted therapy, where effective tools both in preoperative staging and in peri-operative treatment (neoadjuvant and adjuvant therapy) are widely available, the importance of an accurate prognostication is crucial for the best possible management of gastric cancer patients.

A cancer staging system should describe the severity of a neoplasm according to the extent of primary tumor and of its spread, both local and distant; this allows clinicians to calculate prognosis, to compare groups of patients, and to determine the treatment strategy. As it should be used in an everyday clinical practice setting, an ideal cancer staging system has to be easily reproducible and applicable, both before and after treatment.

Since its first application in the staging of gastric malignancies in 1974^[2], the tumor-node-metastasis (TNM) staging system has been periodically modified, in order to ameliorate its accuracy in stratification of prognosis in gastric cancer patients.

The current TNM system for gastric cancer, although still improvable, well fulfills the above mentioned requirements.

In last five years different Authors analyzed usefulness of the seventh TNM classification and, up to now, it seems that different issues still require further improvements.

In this review, we summarize current opinions and controversies on the seventh classification of the TNM staging system for gastric cancer, introducing open questions and new issues which may integrate the traditional way of staging.

LITERATURE RESEARCH

Recent Literature (from 2010 up to March 2016) was

evaluated on PubMed Central with combination of following MESH terms: gastric cancer and staging system, gastric cancer and TNM, esophago-gastric junction and staging, gastric cancer and distant metastasis. All abstracts were read separately by two different surgeons belonging to an Italian University Hospital, and scientific relevance of papers has been assessed mostly according to originality of the article, accuracy of the statistical method and number of patients. All of the selected papers were fully read by two or more surgeons, and only papers reported in References have been judged clinically and scientifically relevant.

ADENOCARCINOMA OF ESOPHAGO-GASTRIC JUNCTION

Latest TNM classification made an effort to clarify recommendations about malignancies arising at or close to the esophago-gastric junction. According to anatomical criteria consistent with the 5-cm rule of Siewert Classification^[3], seventh TNM classification included esophago-gastric junction tumor in the esophageal chapter^[4]. Unfortunately, this proposal might lead to classify as esophageal a tumor of the gastric fundus^[5]. Hence, most Adenocarcinoma of esophago-gastric junction (AEGs), including adenocarcinomas of the cardia and subcardia, are now to be staged as esophageal adenocarcinomas rather than gastric cancers, although they actually originate from the gastric mucosa and consequently have different biological properties compared with genuine gastric and genuine esophageal cancers^[6]; therefore cardia tumors still remain in a no-man's land of staging.

Actually, the esophago-gastric junction is a peculiar transitional area from squamous to glandular epithelium, which is different from epithelium of distal stomach. Concerning macroscopic anatomy, the intra-abdominal part of the esophagus, esophagogastric junction, and fundus are not totally covered by visceral peritoneum. These portions of the stomach are located extraperitoneally or retroperitoneally, which makes AEG more prone to infiltrate the serosa and more inclined to peritoneal metastasis; moreover, a different pathway of lymphatic metastases has to be considered^[7].

These anatomical differences related to the esophago-gastric junction imply a different oncological management as well as a different surgical approach than gastric or esophageal cancer: basically, the clinicians should early know the correct strategy and a reliable prognosis to present to their patients.

Although some authors reported better prognostication when AEG II/III tumors were staged as gastric cancer^[8,9], several studies advocate the introduction of a separate staging system for neoplasm of this "damned" anatomical district, as neither the esophageal

nor the gastric staging scheme could well stratify the prognosis of these patients^[10-12].

Staging of tumors arising from the esophagogastric junction should require further revision.

Distant metastasis (M)

Regarding assessment of M parameter, Mx has been deleted. To be correct, the lack of information about status of anatomical districts far from primary tumor site must be considered as inappropriate.

In every day clinical practice, it means that an accurate staging process must be performed (both before and after treatment); this again, became even more important, since latest TNM classification included findings of positive peritoneal cytology (as well as omental tumor not part of continuous extension) in M1 category^[4].

After more than five years since this proposal, unfortunately, clinical practice has not been really modified: in fact, the routinely use of laparoscopic staging, which allows both retrieval of free fluid for cytologic examination and inspection of the entire peritoneal cavity, and thus eventual omental implants^[5,13], has not reached a wide diffusion yet^[14]. This "bad habit" is responsible for understaging of disease or useless laparotomies in about 20% of cases^[15,16].

Moreover, since some studies reported outcomes of potentially curative resections after the clearance of peritoneal cytology (conversion from positive to negative after neoadjuvant chemotherapy)^[17,18], it might be responsible of sub-optimal treatment.

Actually, a standardization of the method used to perform peritoneal cytology is needed: in fact, different rates of positive cases with different techniques have been reported: > 20% on a routine cytology, 35% on immunohistochemistry and 50% on RT-PCR in cases of a serosa invasion-positive gastric carcinoma^[19].

This new change in the last TNM classification could be considered an improvement according to prognostic results. Nevertheless, it should suggest a more reliable compliance to clinicians.

Node (N)

The cut off of metastatic regional lymph nodes in the N category was changed, too (N1 = 1-2 nodes; N2= 3-6 nodes; N3a= 7-15 nodes; N3b= more than 15 nodes)^[4]. Moreover, the minimum number of required nodes reached 16, although this seems to be in contrast with the sentence (added in the previous edition) which allows to classify as pN0 also negative nodes tumors even if the minimum number of examined nodes is not met. This proposal seems to derive from the need to minimize non-homogeneity in the extent of lymphadenectomy.

Actually, lymph node staging is the main object of current controversies in TNM staging system. In last years, different authors compared the prognostic

power between the sixth and the seventh TNM classification, as well as they proposed possible alternative staging systems.

According to comparison studies, seventh TNM classification for gastric cancer provided a more detailed classification of prognosis than the sixth system^[20,21]. With specific regard to the proposal of new staging criteria, lymph node ratio (LNR) gained increasing popularity. It is defined as the ratio between the number of positive nodes and the number of total examined nodes. Most studies concluded that LNR is superior to the traditional N stage in TNM system in stratifying the prognosis of gastric cancer patients^[22-28]. LNR, and other alternative node staging system, such as LODDs (log of the ratio between the probability of being a positive lymph node and the probability of being a negative lymph node when one lymph node is retrieved)^[29], have been proposed especially in groups of patients with less than 15 retrieved nodes: thus, it seems that most of the attempts are made to justify a suboptimal surgery about lymphadenectomy, rather than to increase prognostic power of pathological lymph node staging^[30,31].

In this context, a possible further staging improvement could be to associate LNR (instead of the more complex LODDs) with the last numeric criterion.

Tumor (T)

Regarding the T parameter, latest TNM classification has introduced high grade dysplasia (HGD) in Tis category; T1 category has been subdivided into T1a (tumors involving lamina propria or muscularis mucosae) and T1b (tumors involving submucosa), and T2b has been replaced with new T3 category, so that serosal involvement is now considered a T4 tumor (T4a category differently from T4b category assigned to tumors invading adjacent structures)^[4].

Inclusion of HGD in Tis category lead to a more aggressive approach to this histologic entity. According to latest Japanese guidelines for gastric cancer^[32], some histologic and dimensional criteria have to be met in order to perform endoscopic mucosal resection (EMR) or endoscopic submucosal dissection: therefore, preoperative diagnosis has to be even more accurate.

Furthermore, as distinguishing invasive carcinoma from HGD in gastric biopsy specimens is not always possible, EMR can also be proposed in order to obtain a more accurate histologic definition^[33].

Finally, new T3 category implied for the first time the definition of locally advanced disease for a tumor entirely contained into the gastric wall. As subserosal involvement may not be correctly evaluated during staging laparoscopy, EUS - which remains the first-choice imaging modality in preoperative T staging of gastric cancer - emerges again as a crucial step in pre-treatment staging^[34]. Nonetheless, it is not always included in staging algorithm. De facto, although trial about perioperative chemotherapy have been

Table 1 Stage grouping based on cluster analysis in the International gastric cancer association stage grouping

	N0	N1	N2	N3a	N3b
T1	I A	I B	II A	II B	III B ¹
T2	I B	II A	II B	III A	III B ¹
T3	II A	II B	III A	III B	III C ¹
T4a	II B	III A	III A ¹	III B ¹	III C
T4b	III A ¹	III B	III B ¹	III C	III C

¹Categories are different from 7th TNM grouping.

conducted even on T2 tumors^[35], at the moment T3 is often excluded from perioperative treatment strategy.

The change of Tis and T1 categories simplified the (endoscopic) management of early cancers. Even if the change of T3 category did not imply significant publications on the comparison between the sixth and the seventh TNM classification, in the future revisions it could be useful to reconsider a simplification of the staging related to the deep involvement of stomach wall.

GROUP STAGING

Finally, the aforementioned revisions of the three parameters also resulted in a rearrangement of stage grouping. The most important feature in latest TNM is that only distant metastasis defines stage IV (very poor prognosis)^[41]; this means that T4 and N3 are not necessarily considered signs of significantly advanced disease anymore. This could probably have been induced by an excessive optimism towards multimodal treatment, recruiting at the moment more advanced disease^[36]. However, patients with N3b tumors do have a very scarce prognosis^[37], although they are considered same as N3a in stage grouping.

Dikken *et al.*^[38], testing latest TNM in prognostication of 2196 patients, found a decreased heterogeneity among stage groups, and observed that the increased complexity of the latest TNM is not accompanied by an improvement in prognostic accuracy of stage grouping. Regarding this aspect, Röcken *et al.*^[39] proposed to reduce to 3 groups (instead of 7) M0 patients according to different combinations of the new T and N parameters: low risk group with > 60% 5-year survival rate, intermediate risk group with 20%-60% 5-year survival rate and high risk group with < 20% 5-year survival rate.

Since stage grouping is essential in prognostication (not in treatment planning), hopefully summing the three parameters, it is probably here that great simplicity and high accuracy has to be reached.

CONCLUSION

Although many criticisms have been reported since seventh TNM classification was adopted, we may consider it a valuable tool in assessing the extent of

disease, and a good instrument in everyday clinical practice.

The International Gastric Cancer Association (IGCA) launched in 2009 a staging project with the aim of collecting gastric cancer data worldwide, in order to formulate a contemporary evidence based classification. This project collected data from 59 Institutions in 15 Countries and used them to validate the latest TNM classification: for both T and N it accepted the categories of the seventh TNM and then assessed a new stage grouping scheme (Table 1), which better stratifies prognosis also in patients with Siewert type II or III tumor. A relevant aspect of this new staging system is the split between N3a and N3b categories, derived from finding of worse survival in patients with more of 6 involved nodes. This new scheme could be introduced in next TNM classification^[29] along with some implications of the above mentioned considerations: hence, this next staging solution, tested with data from both Eastern and Western patients, should allow to appropriately compare treatment results in different regions.

If the IGCA staging system at the moment does not add any new parameter, other Authors reflected on possible introduction of more details to improve accuracy of prognostication.

As specified, the role of LNR seems to be quite widely accepted, but other issues regarding lymph node involvement are still debated, such as the presence of isolated tumor cells, now classified as pN0(i+), which might be classified as pN1(i+), and the extra capsular extension of regional metastasis in adjacent tissues^[39].

Again, lymphatic and venous invasion have been proposed for a better definition of T category^[40], as well as the possible use of molecular findings (*i.e.*, HER2), but it is still to determine whether these data should be actually introduced in the TNM staging system or they should be considered as additional (and thus optional) prognostic factors.

In conclusion, further improvements are obviously needed, but if on one hand the integration with molecular and histopathological findings may give more precision to prognosis prediction, on the other hand it will certainly reduce the easy applicability of the staging scheme^[39].

Without any doubt we are going toward a more specific and precise staging system, and this requires strict statistical evaluations. While waiting for the perfect staging system, we should at first reach standardization of both surgical (use of staging laparoscopy, extent of resection, proper lymphadenectomy) and pathological technique (blocks of primary tumor, nodes count accuracy, immunoistochemistry of lymph nodes to detect isolated tumor cells, technique for peritoneal cytology), so that the future TNM classification will not be conditioned by the need of supply to inadequate surgery and/or inadequate pathological staging.

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Sessile serrated adenoma/polyps: Where are we at in 2016?

Rajvinder Singh, Leonardo Zorrón Cheng Tao Pu, Doreen Koay, Alastair Burt

Rajvinder Singh, Gastroenterology Department, Lyell McEwin and Modbury Hospital, NALHN School of Medicine, University of Adelaide, SA 5112, Australia

Leonardo Zorrón Cheng Tao Pu, Gastrointestinal Endoscopy Division, Department of Gastroenterology, Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo, São Paulo 05403-000, Brazil

Leonardo Zorrón Cheng Tao Pu, Gastroenterology Department, Lyell McEwin and Modbury Hospital, University of Adelaide, SA 5000, Australia

Doreen Koay, Gastroenterology Department, Lyell McEwin and Modbury Hospital, University of Adelaide, SA 5000, Australia

Alastair Burt, Executive Dean, Faculty of Health Sciences, University of Adelaide, SA 5000, Australia

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Correspondence to: Rajvinder Singh, MBBS, MPhil, FRACP, AM, FRCP, Chair of the AGEA, Director of Gastroenterology, Clinical Associate Professor, Lyell McEwin and Modbury Hospital, NALHN School of Medicine, University of Adelaide, Haydown Road Elizabeth Vale, SA 5112,

Australia. rajvinder.singh@sa.gov.au
Telephone: +61-8-81829909
Fax: +61-8-81829837

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Abstract

It is currently known that colorectal cancers (CRC) arise from 3 different pathways: the adenoma to carcinoma chromosomal instability pathway (50%-70%); the mutator "Lynch syndrome" route (3%-5%); and the serrated pathway (30%-35%). The World Health Organization has classified serrated polyps into three types of lesions: hyperplastic polyps (HP), sessile serrated adenomas/polyps (SSA/P) and traditional serrated adenomas (TSA), the latter two strongly associated with development of CRCs. HPs do not cause cancer and TSAs are rare. SSA/P appear to be the responsible precursor lesion for the development of cancers through the serrated pathway. Both HPs and SSA/Ps appear morphologically similar. SSA/P are difficult to detect. The margins are normally inconspicuous. *En bloc* resection of these polyps can hence be troublesome. A careful examination of borders, submucosal injection of a dye solution (for larger lesions) and resection of a rim of normal tissue around the lesion may ensure total eradication of these lesions.

Key words: Colonoscopy; Sessile serrated adenoma/polyp; Serrated lesion; Colorectal polyps; Colorectal cancer; Polypectomy; Image enhancing endoscopy; Narrow band imaging, Endocytoscopy

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Core tip: Colorectal cancers (CRC) arise from 3 pathways: adenoma to carcinoma; "Lynch syndrome"; and serrated. There are 3 types of serrated lesions namely: Hyperplastic Polyps, Sessile Serrated Adenomas/Polyps and Traditional Serrated Adenomas, the latter two are associated with CRC. A careful examination of borders, submucosal injection with dye and ensuring that a rim of normal tissue is removed is paramount.

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INTRODUCTION

Colorectal cancer (CRC) is a major health concern, especially in western countries. According to the American Cancer Society's estimates, CRC accounts for almost 50000 deaths in the United States with almost 130000 new cases diagnosed in 2016. It is the third commonest type of cancer. Effective screening programs for identification of malignant and premalignant colorectal lesions are thus of utmost importance. In the last few decades the adenoma to adenocarcinoma pathway has been well recognized. For some time it was believed to be the only pathway apart from the "Lynch syndrome" route that results in the development of CRC. The effort to detect and eradicate adenoma have been the main goal in preventive colorectal programs, leading to improved outcomes. Zauber *et al*^[1] showed that colonoscopic removal of adenomatous polyps led to a 53% reduction in mortality from CRC during the first 10 years after polypectomy.

It is currently believed that CRC arises from 3 different pathways: the adenoma to carcinoma pathway which accounts for about 50%-70% of cancers; through the mutator "Lynch syndrome" route (3%-5%); and more recently the serrated pathway (30%-35%). The latter have become increasingly recognized as a separate route which could lead to the development of CRC^[2].

This triplet division is based on the combined clinical-molecular characteristics of the lesions. A deeper understanding of the molecular pathways in CRC have been described by Jass in 2007^[3] and updated by Phipps *et al*^[4] in 2015. They described 5 molecular subtypes and associated genetic distortions to describe each one. Subtypes 1, 2 and 3 are related to the serrated pathway. Subtypes 1 and 2 are either microsatellite instable (MSI)-high or microsatellite

stable (MSS)/MSI-low cancers which have the CpG island methylator phenotype (CIMP) and *BRAF*-mutation but are *KRAS* negative. The third subtype represents an alternative pathway which originates in *KRAS* mutation with no CIMP, *BRAF* or MSI association. Subtypes 2 and 3 have a higher association with mortality^[4]. Subtype 4 reflects CRC arising from the traditional adenoma-carcinoma sequence, and are MSS/MSI-low, CIMP, *BRAF* and *KRAS* negative. Subtype 5 indicates Lynch syndrome and is associated with high prevalence of a family history of CRC. They are MSI-high but CIMP, *BRAF* and *KRAS* negative.

The serrated pathway is much less well understood. Systematic resection of premalignant serrated lesions could further improve the outcomes of CRC screening programs. One of the main problems with this protocol is the difficulty in identifying these lesions. Unlike adenomas, not all serrated lesions are linked to colorectal cancer. According to the World Health Organization, there are three types of serrated lesions: Hyperplastic polyps (HP), sessile serrated adenomas/polyps (SSA/P) and traditional serrated adenomas (TSA). TSA is usually easy to identify due to its protuberant pine cone-shape. While SSA/P is also associated with cancer, HP is not and their discrimination is troublesome as they look morphologically similar at colonoscopy, even with image enhancing endoscopy (IEE) techniques. Despite the adoption of numerous different classifications, the ability to predict HP from SSA/P has unfortunately been overlooked^[5,6]. More recently, a newly proposed approach known as Workgroup Serrated polypS and Polyposis WASP classification has allowed the distinction between HP and SSA/P with reasonable accuracy^[7]. It consists of cloud-like surface, indistinctive borders, irregular margins and open pit patterns, features described as being associated with SSA/P in another previous study^[8]. The need to adequately identify SSA/P from HP arises from evidence supporting SSA/P as the major malignant source amongst serrated lesions^[2,9-12].

IEE

Detecting and characterizing colorectal lesions by IEE has been reported in several articles^[13-17] and has been found to have 92.7% sensitivity and 87.3% specificity in differentiating adenomas/adenocarcinomas from "non-neoplastic" lesions^[18]. Differentiating serrated lesions, specifically SSA/P from HP is more challenging. The incidence of serrated lesions in the overall population is 5%-8% (contrasting with 30%-40% for adenomas), and they are more difficult to see due to their colour and shape^[8,19,20]. Their rarity and discreet morphology could be why there is a longer learning curve compared to that for adenomas^[21-24].

The evaluation of dysplasia within the SSA/Ps could also be of value. It has been described by Chino *et al*^[25] 2016 that the evaluation of crypts and submu-

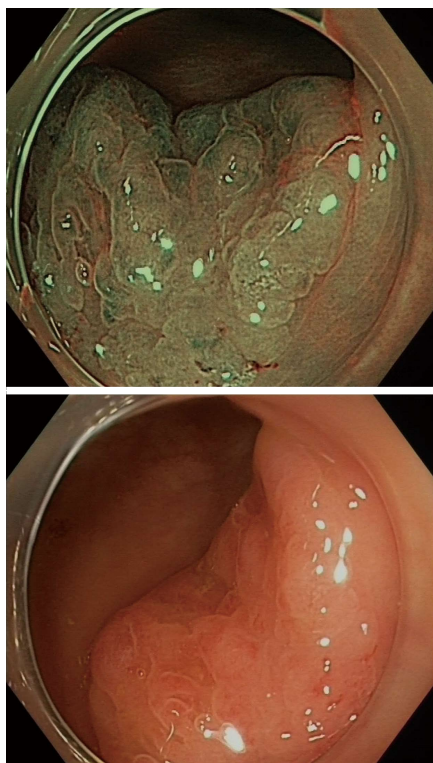


Figure 1 Inconspicuous margins of a sessile serrated adenomas/polyps with and without narrow-band imaging.

cosal vessels with narrow band imaging (NBI) and magnification might be useful in evaluating dysplasia in SSA/P, which leads to poorer outcomes.

Although there is certainly enthusiasm for IEE techniques, histopathology remains the gold standard for evaluating colorectal lesions. Nonetheless, improving technology that could be used by the endoscopist in real time would definitely be beneficial for serrated lesions as it has been for adenomas^[26]. This technology will need to provide immediate feedback and accurately predict the final histopathology (Figure 1).

SSA/P AND HP DIFFERENTIATION

A conceptual way to define each serrated lesion is based on differences in the proliferation zones within the serrated crypts in each group^[27]. In HP, the expanded proliferation zone is located at the base of the crypts and cells mature towards the surface symmetrically. In SSA/P, the proliferation zone is to the side of the crypts instead of the base, resulting in maturation of epithelial cells laterally, towards the surface and the base, leading to crypt base dilatation (pattern II -open). Within SSA/P, the presence of dysplasia is usually evident and must be accompanied by SSA/P component adjacent to it once its histopathology is similar to adenomas. Unfortunately, this theoretical classification may be misleading. Confounded even by expert pathologists, the poor agreement for the diagnosis of villous features or high

grade dysplasia has a 10-fold variability^[28-30].

New techniques for real-time *in vivo* optical diagnosis using IEE have been developed to potentially predict histology and perhaps permit a more practical and economical approach for low-risk polyps; for example the "resect and discard" approach^[31-34]. There is evidence from several original articles and meta-analyses that *in vivo* optical diagnosis using either NBI or Fujinon intelligent chromoendoscopy would be more cost-effective compared to histology without significant changes in follow-up decision, especially for diminutive polyps^[34-37]. The American Society for Gastrointestinal Endoscopy statement of 2011 (Preservation and Incorporation of Valuable endoscopic Innovations) describes the standards that new technologies have to achieve in order to be implemented. For the "resect and discard" strategy, it asks for $\geq 90\%$ agreement in the assignment of post-polypectomy surveillance intervals when compared with decisions based on histopathology. With regards to the policy of leaving suspected rectosigmoid hyperplastic polyps measuring ≤ 5 mm in place, a $\geq 90\%$ negative predictive value for adenomatous histology is mandated^[31]. Abu Dayyeh *et al*^[38] on behalf of the American Societies for Gastrointestinal Endoscopy (ASGE) Technology Committee in 2015 reported in a meta-analysis that the diagnostic value of IEE for diminutive colorectal polyps achieved a pooled NPV 91% and pooled follow-up agreement of 89%. Despite the pooled analysis for agreement in the assignment of surveillance intervals which did not reach the 90% threshold for NBI; experienced endoscopists were able to exceed this (93%) when the diagnosis was made with high confidence.

PREDICTORS OF MALIGNANCY AMONG SSA/P

The most common group of lesions are the diminutive polyps (≤ 5 mm in size), which represent approximately 60% of all polyps detected at primary screening colonoscopy. Their overall association with advanced pathology is low but not negligible^[39,40]. On the contrary, Burgess *et al*^[41] have demonstrated that size matters in terms of SSA/P. For every 10 mm increase in lesion size, the OR is 1.90 for cytological dysplasia. SSA/P with cytological dysplasia (SSA/P-D) is also associated with presence of 0-Is component of the Paris' Classification (OR = 3.1), Kudo's pit pattern III, IV or V (OR = 3.98) and increasing age (OR = 1.69 per decade).

Yamada *et al*^[32] recently described the presence of dilated branch vessels as an aspect of SSA/Ps with dysplasia. Apart from their characteristics at chromoendoscopy and magnification^[42,43], there are some aspects that we can use to distinguish SSA/Ps with and without malignancy potential.

Endocytoscopy is an emerging modality with

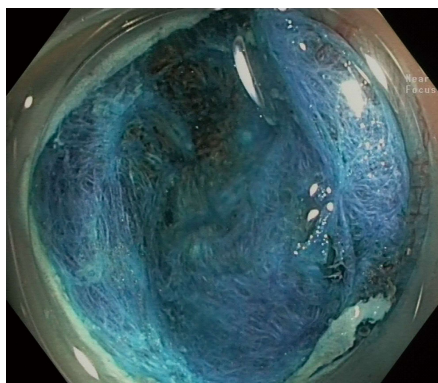


Figure 2 Resection of a sessile serrated adenomas/polyps with dye of submucosal layer with indigo carmine - no residual lesion.

diagnostic potential for SSA/P. It allows *in vivo* visualization of cells and nuclei facilitating precise real-time pathological prediction. Oval gland lumens with small round nuclei has a sensitivity of 83.3% and specificity of 97.8% for the diagnosis of SSA/P. It is also a promising tool for diagnosing SSA/P-D due to its ability to detect morphological changes in the nuclei as described by Mori *et al.*^[44] and Kutsukawa *et al.*^[45].

OPTIMAL RESECTION OF AN SSA/P

Numerous studies have grim numbers in regards to SSA/P complete resection rates^[46-48]. Against these odds, a more recent study from our group^[49] studied the resection of 2000 lateral spreading tumors and attributed the high recurrence to the inconspicuous margins of the SSA/P, which was overcome with IEE techniques. Submucosal instillation of a dye based solution (for larger lesions), a careful examination of borders and a rim of normal tissue resected together with the lesion may have affected the high rate of complete removal of the SSA/P. It is evident the contribution that advanced endoscopy apparel and endoscopist's expertise is essential^[50] in order to keep the recurrence of resection as low as 7%, as described by Pellise *et al.*^[49] (Figure 2).

FOLLOW-UP

The current guidelines from the ASGE and European Societies for Gastrointestinal Endoscopy advocates the standard 5-10 years surveillance period for low risk lesions (SSA/P < 10 mm and without dysplasia), in patients without serrated polyposis syndrome. Patients with larger SSA/Ps or with dysplasia should have their colonoscopy repeated in 3 years-time^[51,52]. The serrated polyposis syndrome is defined if any number of serrated polyps occurring proximal to the sigmoid colon in an individual who has a first-degree relative with serrated polyposis; if at least five serrated polyps are found proximal to the sigmoid colon (at least 2 with ≥ 10 mm); and if more than 20 serrated

polyps of any size distributed throughout the colon. In these cases, the follow-up should be at 1 year^[51]. The major problem is that these guidelines rely upon the assumption that the serrated lesions are detected and resected adequately, which is not always the case.

CONCLUSION

SSA/P is an important pre-malignant lesion that can easily be missed. Efforts must be made in order to alter the nomenclature of "non-neoplastic lesions" to non-adenomatous lesions as the role of serrated lesions in the development of colorectal cancer is now well established. A longer training must be pursued and cutting-edge IEE technologies developed and studied in order to diminish the miss rate for serrated lesions. The implementation of a "serrated polyps detection rate" could be implemented alongside the "adenoma detection rate" as a quality indicator for colonoscopy.

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Long-term outcomes of autoimmune pancreatitis

Tsukasa Ikeura, Hideaki Miyoshi, Masaaki Shimatani, Kazushige Uchida, Makoto Takaoka, Kazuichi Okazaki

Tsukasa Ikeura, Hideaki Miyoshi, Masaaki Shimatani, Kazushige Uchida, Makoto Takaoka, Kazuichi Okazaki, Department of Gastroenterology and Hepatology, Kansai Medical University, Hirakata, Osaka 573-1010, Japan

Author contributions: Ikeura T, Miyoshi H and Shimatani M wrote the manuscript; Uchida K and Takaoka M made revision of the manuscript; Okazaki K made final approval of the manuscript to be published.

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Correspondence to: Tsukasa Ikeura, MD, PhD, Department of Gastroenterology and Hepatology, Kansai Medical University, 2-3-1, Shinmachi, Hirakata, Osaka 573-1010, Japan. ikeurat@hirakata.kmu.ac.jp
Telephone: +81-72-8040101
Fax: +81-72-8042534

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Abstract

Autoimmune pancreatitis (AIP) has been considered a favorable-prognosis disease; however, currently, there is limited information on natural course of

AIP during long-term follow-up. Recently published studies regarding the long-term outcomes of AIP has demonstrated the developments of pancreatic stone formation, exocrine insufficiency, and endocrine insufficiency are observed in 5%-41%, 34%-82%, and 38%-57% of patients having the disease. Furthermore, the incidence rate of developing pancreatic cancer ranges from 0% to 4.8% during the long-term follow-up. The event of death from AIP-related complications other than accompanying cancer is likely to be rare. During follow-up of AIP patients, careful surveillance for not only relapse of the disease but also development of complications at regular intervals is needed.

Key words: Autoimmune pancreatitis; Outcome; Cancer; Prognosis

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Core tip: There is limited information on long-term outcomes of patients with autoimmune pancreatitis (AIP). This review provides a current overview of AIP regarding long-term outcomes such as pancreatic stone formation, pancreatic exocrine or endocrine dysfunction, associated malignancy, and mortality.

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INTRODUCTION

Autoimmune pancreatitis (AIP), which was first proposed as a novel clinical entity by Yoshida *et al*^[1] in 1995, is a unique chronic inflammation of the pancreas^[2]. The disease is radiologically characterized by focal or diffuse pancreatic enlargement and irregular narrowing of

Table 1 Development of pancreatic stone formation and functional impairment in autoimmune pancreatitis patients during long-term follow-up

Ref.	Year	Follow-up period (mo)	Incident rate		
			Pancreatic stone	Endocrine dysfunction	Endocrine dysfunction
Uchida <i>et al</i> ^[15]	2006	41	4.8% (1/21)	60.0% (6/10)	46.2% (6/13)
Maire <i>et al</i> ^[23]	2011	50	NA	34.1% (15/44)	38.6% (17/44)
Maruyama <i>et al</i> ^[14]	2012	91	40.6% (28/69)	NA	NA
Hart <i>et al</i> ^[12]	2013	NA	7.0% (46/659)	NA	NA
Hirano <i>et al</i> ^[13]	2013	76	11.3% (8/71)	NA	NA
Buijs <i>et al</i> ^[24]	2015	75	NA	82.4% (56/68)	56.1% (37/66)

NA: Not available.

the main pancreatic duct^[3]. The main clinical finding is a dramatic response to steroids. Two histological subtypes of AIP have been recognized, namely types 1 and 2^[3-6]. Type 1 AIP is histologically characterized by periductal abundant infiltration of lymphocytes with IgG4-positive plasma cells, storiform fibrosis, and obliterative phlebitis. Patients with type 1 AIP are often elderly men, with elevated serum IgG4 levels and extrapancreatic lesions (e.g., sclerosing cholangitis, sclerosing sialadenitis, and retroperitoneal fibrosis). Recently, type 1 AIP is considered as a pancreatic manifestation of IgG4-related disease (IgG4-RD)^[7,8]. By contrast, the histological findings of type 2 AIP are characterized by the presence of granulocytic epithelial lesions (GEL), but not IgG4-positive plasma cells around the large or medium sized pancreatic duct. Patients with type 2 AIP are often younger, have normal serum IgG4 levels, and frequently develop inflammatory bowel diseases, particularly ulcerative colitis^[9].

Generally, the short-term prognosis of AIP is considered favorable based on the remarkable improvement of clinical and radiological findings after steroid therapy. By contrast, information on the long-term prognosis of AIP is limited because it is approximately 15 years after discovery of high serum IgG4 concentrations in patients with AIP^[10]. This review provides a current overview of AIP regarding long-term outcomes such as pancreatic stone formation, pancreatic exocrine or endocrine dysfunction, associated malignancy, and mortality.

OCCURRENCE OF PANCREATIC STONE AND DYSFUNCTION DURING LONG-TERM FOLLOW-UP IN PATIENTS WITH AIP

Development of pancreatic stone

In ordinary chronic pancreatitis (CP), especially alcohol-induced pancreatitis, pancreatic stone is the most common complication. Pancreatic stone formation results from the hypersecretion of protein from acinar cells and stasis of pancreatic juice^[11]. In type 1 AIP,

newly formed pancreatic stones or increased formation of pancreatic stones during follow-up is observed in 5%-41% of cases, whereas no patient with type 2 AIP develop pancreatic stone formation (Table 1)^[12-15]. In a multinational study, pancreatic duct stones are regarded as a relatively uncommon complication, occurring only in 7% of type 1 AIP patients with follow-up, and pancreatic stone formation occurs more frequently in patients with relapse of the disease at least once than in patients without relapse^[12]. A multivariate analysis by Hirano *et al*^[13] demonstrated that ethanol consumption of > 50 g/d was a significant risk factor of pancreatic stone formation during the clinical course of type 1 AIP (OR = 7.47; 95%CI: 1.093-51.1, $P = 0.040$), indicating that similar to ordinary CP, changes in the pancreatic juice component due to high alcohol consumption may in part contribute to stone formation. By contrast, Maruyama *et al*^[14] reported that the independent risk factor of pancreatic stone formation is not alcohol intake but narrowed Wirsung's and Santorini's ducts at diagnosis of AIP (OR = 4.4; 95%CI: 1.3-15.5, $P = 0.019$). Moreover, residual pancreatic head swelling and/or narrowing of Wirsung's and Santorini's ducts after corticosteroid therapy were more frequently found in patients with newly formed pancreatic stone than in patients without stone. These results indicate that the stone formation in AIP results from stasis of pancreatic juice due to the narrowing of the pancreatic head^[14].

Pancreatic stone is a major cause of pain in ordinary CP, and thus, some patients require pain management including medical treatment, endoscopic treatment, and surgery. By contrast, AIP patient with pancreatic stone seem not to experience chronic pain^[16-18].

Development of pancreatic functional impairment

In the typical long-term course of ordinary CP, pancreatic exocrine and endocrine dysfunctions occur owing to the destruction of acinar and Langerhans islet cells, inducing maldigestion and diabetes mellitus as clinical presentations^[19]. At the time of AIP diagnosis, exocrine and endocrine insufficiencies were observed in 66%-81% and 46%-67% of cases, respectively^[20-22]. After long-term follow-up, 34%-82% and 39%-57%

Table 2 Characteristics of the autoimmune pancreatitis patients with pancreatic cancer whose clinical data were available

Case	Ref.	Year	Age	Sex	Smoking	Alcohol	Diabetes	Location of the PC	Period onset of AIP to PC
1	Inoue <i>et al</i> ^[39]	2006	62	M	Yes	No	Yes	Body	0 (Synchronous)
2	Ghazale <i>et al</i> ^[40]	2007	72	M	NA	NA	NA	Body	60
3	Witkiewicz <i>et al</i> ^[41]	2008	80	M	NA	NA	NA	Head	0 (Synchronous)
4	Motosugi <i>et al</i> ^[42]	2009	59	M	NA	NA	Yes	Body and tail	0 (Synchronous)
5	Matsubayashi <i>et al</i> ^[43]	2009	65	M	No	No	No	NA	0 (Synchronous)
6	Gupta <i>et al</i> ^[48]	2012	73	M	NA	NA	NA	Tail	120
7	Gupta <i>et al</i> ^[48]	2012	69	M	NA	NA	NA	Head	60
8	Hirano <i>et al</i> ^[47]	2014	58	M	No	NA	Yes	NA	119
9	Hirano <i>et al</i> ^[47]	2014	70	M	No	NA	Yes	NA	162
10	Ikeura <i>et al</i> ^[46]	2014	61	F	Yes	No	No	Head	31
11	Ikeura <i>et al</i> ^[46]	2014	39	F	No	No	No	Body	186
12	Ikeura <i>et al</i> ^[46]	2014	80	M	No	No	Yes	Head	67

NA: Not available; AIP: Autoimmune pancreatitis; PC: Pancreatic cancer.

of AIP patients had pancreatic exocrine and endocrine dysfunctions, respectively (Table 1)^[15,23,24].

The multivariate logistic regression analysis by Buijs *et al*^[24] demonstrated that the risk factors of the development of endocrine insufficiency were longer follow-up period (OR = 1.36; 95%CI: 1.11-1.68) and older age at onset (OR = 1.06; 95%CI: 1.01-1.11). Aggravation of glycemic control or new onset of diabetes mellitus during the clinical course of AIP is significantly associated with pancreatic parenchymal atrophy that is observed in approximately one-third of patients after remission induced by steroid therapy^[12,23,25]. High tobacco intake has been associated with the prevalence of diabetes mellitus after long-term follow-up^[26].

Although the reported ameliorating effect of steroid therapy for pancreatic function varies across studies owing to differences in observation period and definition of pancreatic exocrine and endocrine dysfunctions, steroid therapy appears to induce improvement of pancreatic exocrine function, as assessed by the urine exocrine *N*-benzoyl-L-tyrosyl-p-aminobenzoic acid (BT-PABA) test, in 40%-73% of AIP patients and glycemic control in 15%-63% of AIP patients with preexistent diabetes mellitus^[15,21,22,27,28]. Some research studies emphasize that steroid therapy should be performed to preserve insulin secretion at the early stage of AIP or to improve glucose intolerance^[22,28]. However, some cases show new onset of diabetes mellitus as a side effect of steroid therapy.

Does AIP progress to ordinary CP?

To examine whether type 1 AIP can progress to ordinary CP over a long disease course, Maruyama *et al*^[29] evaluated the data of 73 patients with type 1 AIP who underwent long-term follow-up by using the revised Japanese clinical diagnostic criteria for ordinary CP. Of the 73 patients, 16 (22%) fulfilled the diagnostic criteria for CP. Furthermore, because 7% of the patients with previously diagnosed alcoholic or idiopathic CP had elevated serum IgG4 levels, the authors were concerned about the possibility that

some of the patients with advanced-stage AIP were misdiagnosed as ordinary chronic pancreatitis^[30].

DEVELOPMENT OF PANCREATIC AND EXTRAPANCREATIC CANCERS IN AIP

Chronic inflammatory processes play a role in carcinogenesis in various organs, such as liver cancer in chronic hepatitis B and C infections^[31], gastric cancer in *Helicobacter pylori*-induced gastritis^[32], colon cancer in inflammatory bowel disease^[33], cholangiocarcinoma in primary sclerosing cholangitis^[34], and pancreatic cancer (PC) in ordinary CP^[35]. In type 1 AIP, persistently high IgG4 serum concentrations were observed in 60% of patients even after steroid therapy^[36], and the relapse rate of type 1 AIP is relatively high, reaching up to 31%-57%^[12,37,38]. This suggests the existence of persisting pancreatic inflammation during an apparent clinical remission. Therefore, it is reasonable to assume that type 1 AIP also carries the risk for pancreatic carcinogenesis. Indeed, several case reports^[39-44] and cohort studies^[12,37,45-49] reported that AIP cases synchronously or metachronously develop PC. Of the AIP patients who developed PC, 12 had partly or fully available clinical information, and their characteristics are shown in Table 2. The mean age of the 12 patients was 65.7 years (range, 39-80 years). Five (63%) of 8 patients had diabetes mellitus before the diagnosis of PC. PC was found simultaneously with AIP in 4 patients and developed in 8 patients during the mean follow-up period of 100.6 mo (range, 31-186 mo).

Although the accurate prevalence of the development of PC in AIP is currently unclear because the clinical profile of patients and surveillance strategy of AIP during follow-up differ depending on published studies, the incidence rate of developing PC ranges from 0% to 4.8% during the follow-up period of 33-75 mo^[12,24,27,37,45-52] (Table 3). In an international multicenter analysis, 5 (0.8%) of 659 patients with type 1 AIP were reported to develop PC more than 3 years after the diagnosis of AIP, with the exception of one patient^[12]. Whether the risk of pancreatic

Table 3 Development of cancer at or after autoimmune pancreatitis diagnosis

Ref.	Year	Follow-up period (mo)	Incident rate of PC	Incident rate of extrapancreatic cancer
Nishino <i>et al.</i> ^[27]	2006	41	0.0% (0/12)	16.7% (2/12)
Takuma <i>et al.</i> ^[50]	2011	40	0.0% (0/50)	NA
Yamamoto <i>et al.</i> ^[51]	2012	37	0.0% (0/106) ¹	10.1% (11/106) ¹
Shiokawa <i>et al.</i> ^[52]	2012	40	0.0% (0/108)	13.9% (15/108)
Hart <i>et al.</i> ^[12]	2013	NA	0.7% (5/659)	7.0% (46/659)
Gupta <i>et al.</i> ^[48]	2013	49	2.4% (2/84)	NA
Hart <i>et al.</i> ^[49]	2014	43	0.9% (1/116)	9.5% (11/116)
Huggett <i>et al.</i> ^[37]	2014	33	0.9% (1/115) ²	7.0% (8/115) ²
Hirano <i>et al.</i> ^[47]	2014	73	2.1% (2/95)	11.5% (13/113) ¹
Ikeura <i>et al.</i> ^[46]	2014	62	4.8% (3/63)	3.2% (2/63)
Shimizu <i>et al.</i> ^[45]	2015	54	1.2% (1/84)	11.9% (8/84)
Buijs <i>et al.</i> ^[24]	2015	75	0.0% (0/68)	11.8% (8/68)

¹Includes patients with systemic IgG4-related disease without autoimmune pancreatitis; ²Includes patients with IgG4-related sclerosing cholangitis. NA: Not available; PC: Pancreatic cancer.

and extrapancreatic cancer is increased in patients with AIP compared with the general population is controversial. Japanese studies have demonstrated that the standardized incidence rate for cancers in IgG4-related diseases or AIP ranged from 2.7 to 3.8^[51,52]. In a United Kingdom cohort, the odds ratio of developing cancer at diagnosis or during follow-up was identified to be 2.25 times greater among patients with AIP/IgG4-related sclerosing cholangitis than among patients with age- and sex-matched national statistical data (95%CI: 1.12-3.94, $P = 0.02$)^[37]. By contrast, some studies reported that the risk of developing cancer during follow-up in patients with AIP is comparable with that in patients without AIP^[24,47]. To clarify whether AIP patients are more susceptible to pancreatic or extrapancreatic cancer, a well-designed multicenter study is needed to eliminate various biases.

Few studies provide histological and biological evidence to support the likelihood of developing PC in type 1 AIP. Gupta *et al.*^[48] focused on pancreatic intraepithelial neoplasia (PanIN), widely recognized as the precursor lesion of invasive ductal carcinoma, arising within the pancreases resected from AIP patients. They demonstrated that the prevalence rates of PanIN-1, PanIN-2, and PanIN-3 in AIP patients were 82%, 25%, and 4%, respectively. These rates are comparable with those in ordinary CP, which is a well-established risk factor of PC. In our previous study^[46], 2 patients with type 1 AIP who developed accompanying PC had no PanIN lesion in the non-cancerous region. However, one patient histologically exhibited marked lymphoplasmacytic infiltration with severe fibrosis around the PC, suggesting that carcinogenesis can result from LPSP, as addressed by Motonaga *et al.* Meanwhile, in a genetic research by Kamisawa *et al.*^[53], *K-ras* mutation, an essential factor in the development of pancreatic ductal

adenocarcinoma^[54,55], was identified in the pancreases of all patients with AIP, whereas 40% of patients with chronic alcoholic pancreatitis showed a *K-ras* mutation. These results provide the possibility that AIP is a risk factor of pancreatic carcinogenesis.

Another mechanism was proposed regarding the accompanying cancer in AIP. In a multicenter cohort study by Shiokawa *et al.*^[52], 14% of AIP patients developed several extrapancreatic cancers, including gastric cancer, lung cancer, lymphoma, prostatic cancer, colon cancer, bile duct cancer, and thyroid cancer, during the follow-up period, whereas none of the patients developed PC. Approximately half of these cancers were diagnosed simultaneously with AIP. The detection rate of concurrent cancers at the diagnosis of AIP was significantly higher than those of any cancers in the control population consisting of individuals who underwent for the first time a medical checkup with full examinations. In this cohort, the relative risk of cancer at AIP diagnosis was 4.9 (95%CI: 1.7-14.9). Moreover, most of the patients with cancer diagnosed prior to the diagnosis of AIP did not experience AIP relapse after successful treatment of their cancers. Based on these results, they proposed that AIP may be a manifestation of paraneoplastic syndrome, which is a rare condition triggered by an altered immune system response to a neoplasm^[56]. To clarify the question, "Which comes first, AIP or cancer," further epidemiological data are needed.

In the patients with AIP accompanied by PC, tumors are incidentally discovered as new findings such as mass formation and stricture of the lower bile duct on imaging examination performed as surveillance for AIP relapse. These findings may lead to misdiagnosis as AIP relapse because of the resemblance of the two diseases. In case the development of cancer is suspected in the followed-up patients with AIP, in addition to the assessment of serum CA19-9 level, pathological examination using endoscopic ultrasonography-guided fine-needle aspiration and endoscopic retrograde cholangiopancreatography should be performed.

OUTCOMES OF LONG-TERM MAINTENANCE STEROID THERAPY

Relapses of type 1 AIP more frequently occur during follow-up, compared to type 2 AIP. An international analysis demonstrated relapse rate in type 1 AIP was significantly higher than that in type 2 AIP (31% vs 9%, $P < 0.001$)^[12]. The Japanese consensus guidelines for AIP proposed steroid maintenance therapy (2.5-5 mg/d) within 3 years to prevent relapse of the disease, whereas steroid therapy protocol without maintenance therapy is common in Western countries^[57]. Most recently, Hirano *et al.*^[58] prospectively investigated outcomes after long-term maintenance steroid therapy in 21 patients with AIP. In the study, clinical relapse rate after the cessation of maintenance steroid therapy

was unexpectedly high (48%, 11/21). Based on the results, authors concluded it was desirable to continue maintenance steroid therapy for over 3 years to prevent relapse. However, it is still unknown whether maintenance steroid therapy leads to favorable long-term outcomes not only in terms of prevention of relapse and progression of AIP but also in terms of steroid-related side effects.

AIP-RELATED MORTALITY

Patients with AIP are less likely to die from AIP-related complications other than accompanying cancer, although mortality due to complications of IgG4-related diseases, such as liver and renal failure, has been reported in rare cases^[15,50]. No significant difference in survival was observed between patients with AIP and age- and sex-matched controls from the national population^[24]. When the event of death during follow-up was compared between type 1 and type 2 AIP, the mortality rate in type 1 AIP was significantly higher than that in type 2 AIP^[59]. The explanation for the higher mortality in the patients with type 1 AIP could be partly attributed to their higher age.

Long-term use of maintenance steroid therapy to prevent relapse of the disease can cause serious side effects, which can be fatal and therefore requires considerable attention^[45].

CONCLUSION

Although the characteristics, magnitude, and sequelae of complications that occur during a long-term course of AIP are still poorly understood, careful surveillance for not only relapse of the disease but also development of complications at regular intervals during follow-up of AIP patients is important.

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Basic Study

Tumor necrosis factor- α -G308A polymorphism is associated with liver pathological changes in hepatitis C virus patients

Noha G Bader El Din, Sally Farouk, Reem El-Shenawy, Marwa K Ibrahim, Reham M Dawood, Mostafa M Elhady, Ahmed M Salem, Naglaa Zayed, Ahmed Khairy, Mostafa K El Awady

Noha G Bader El Din, Sally Farouk, Reem El-Shenawy, Marwa K Ibrahim, Reham M Dawood, Mostafa K El Awady, Department of Microbial Biotechnology, National Research Centre, Dokki, Giza 12622, Egypt

Mostafa M Elhady, Ahmed M Salem, Department of Biochemistry, Faculty of Science, Ain Shams University, Cairo 11566, Egypt

Naglaa Zayed, Ahmed Khairy, Endemic Medicine Department, Faculty of Medicine, Cairo University, Giza 11562, Egypt

Author contributions: Bader El Din NG designed and supervised the experiments, analyzed the data and wrote the article; Farouk S performed the research experiments and analyzed the data; El-Shenawy R, Ibrahim MK and Dawood RM helped in DNA extraction and in collecting and analyzing the data; Elhady MM and Salem AM performed the PCR purification and sequence analysis; Zayed N and Khairy A collected the blood samples and patient records; El Awady MK directed the study and provided financial support.

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Correspondence to: Noha G Bader El Din, PhD, Professor of Medical Biotechnology, Department of Microbial Biotechnology, National Research Centre, 33 EL Bohouth Street (Former EL Tahrir Street), Dokki, Giza 12622, Egypt. nbadereldin@yahoo.com
Telephone: +20-2-33371362
Fax: +20-2-33370931

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Abstract

AIM

To investigate the association of tumor necrosis factor alpha (TNF α) -G308A polymorphism with different liver pathological changes in treatment-naïve Egyptian patients infected with hepatitis C virus (HCV) genotype 4.

METHODS

This study included 180 subjects, composed of 120 treatment-naïve chronic HCV patients with different fibrosis grades (F0-F4) and 60 healthy controls. The TNF α -G308A region was amplified by PCR and the different genotypes were detected by restriction fragment length polymorphism analysis. The TNF α protein was detected by enzyme-linked immunosorbent assay. The influence of different TNF α -G308A genotypes on TNF α expression and liver disease progression were statistically analyzed. The OR and 95%CI were calculated to assess the relative risk confidence.

RESULTS

Current data showed that the TNF α -G308A SNP frequency was significantly different between controls and HCV infected patients ($P = 0.001$). Both the AA genotype and A allele were significantly higher in late fibrosis patients (F2-F4, $n = 60$) than in early fibrosis patients (F0-F1, $n = 60$) ($P = 0.05, 0.04$ respectively). Moreover, the GA or AA genotypes increased the TNF α serum level greater than the GG genotype ($P = 0.002$). The results showed a clear association between severe liver pathological conditions (inflammation, steatosis and fibrosis) and (GA + AA) genotypes ($P = 0.035, 0.03, 0.04$ respectively). The stepwise logistic regression analysis showed that the TNF α genotypes (GA + AA) were significantly associated with liver inflammation (OR = 3.776, 95%CI: 1.399-10.194, $P = 0.009$), severe steatosis (OR = 4.49, 95%CI: 1.441-14.0, $P = 0.010$) and fibrosis progression (OR = 2.84, 95%CI: 1.080-7.472, $P = 0.034$). Also, the A allele was an independent risk factor for liver inflammation ($P = 0.003$), steatosis ($P = 0.003$) and fibrosis ($P = 0.014$).

CONCLUSION

TNF α SNP at nucleotide -308 represents an important genetic marker that can be used for the prognosis of different liver pathological changes in HCV infected patients

Key words: Hepatitis C virus immune response; Tumor necrosis factor alpha; Single nucleotide polymorphisms; Cytokine expression; Liver disease progression

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Core tip: Tumor necrosis factor alpha (TNF α) is a proinflammatory and antiviral cytokine that plays a major role in liver injury and initiation of the fibrosis process. We investigated the association of TNF α -G308A polymorphism with liver pathological changes in treatment-naïve Egyptian patients infected with hepatitis C virus (HCV) genotype 4. The results showed that the TNF α A allele produced high circulating TNF α in the body, which induces inflammatory response. The TNF α A allele was an independent risk factor for liver inflammation, steatosis and fibrosis. TNF α -G308A represents an important genetic marker that can be used for the prognosis of different liver pathological changes in HCV infected patients.

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INTRODUCTION

Hepatic fibrosis is the liver wound healing process for many injuries, such as excess vitamin A, inherited metabolic disorders, alcohol, drugs, cholestatic disorders, viral hepatitis and autoimmune disorders^[1]. In Egypt, hepatitis C virus (HCV) is a major public health problem, with a 15% prevalence rate and the predominant genotype being genotype 4^[2]. Chronic HCV patients are usually at risk of developing cirrhosis and hepatocellular carcinoma. Complications of HCV infection in Egypt are responsible for 67% of the liver disease death rate^[3].

Exacerbated immune response against HCV, along with the consequent excess production of chronic inflammatory mediators, is the underlying cause of liver injury and development of fibrosis. Chronically infected patients may have a slow, intermediate or rapid fibrosis progression rate. Multiple factors have been reported to affect the liver disease progression rate^[4]. Single nucleotide polymorphisms (SNPs) in the non-coding or coding regions of cytokine genes have been documented to affect their expression and secretion, which can cause chronic inflammation and disease progression^[5,6].

Altered expression of cytokines, such as tumor necrosis factor alpha (TNF α), transforming growth factor beta, interferon gamma (IFN γ), platelet-derived growth factor, interleukin (IL)-1, -10 and -28B, and the IL-1 receptor antagonist, were found to be associated with liver fibrosis^[1,7,8]. The expression of these cytokines plays important roles in the regulation of liver cell functions and the fibrotic process *in vivo*. TNF α is a proinflammatory and antiviral cytokine secreted by macrophage and cytotoxic T lymphocytes in the liver, where it regulates the immune response, cell growth and apoptosis. TNF α is considered the immune-mediated hepatic cytokine that plays a major role in liver injury and the initiation of the fibrosis process. It stimulates hepatic stellate cells' proliferation, chemoattraction, extracellular matrix components' secretion and connective tissue growth factors' expression. Therefore, TNF α expression is considered a key molecular link between liver inflammation, steatosis and fibrosis^[9,10].

The TNF α gene is located on chromosome 6p21.3^[11]. Six polymorphisms at nucleotides -1031, -863, -857, -376, -308 and -238 in the TNF promoter were thought to influence the TNF α expression^[12,13]. Several studies showed that the TNF α -G308A polymorphism affects the TNF α transcription level. In the normal population, the wild genotype is GG and AA is the mutant genotype. The A allele has a significant functional effect on TNF transcription and is associated with higher inducible TNF α transcription levels than the G allele^[14].

Many inflammatory disorders and infectious

diseases are associated with TNF α -G308A polymorphism, such as systemic lupus erythematosus, inflammatory bowel disease, insulin-dependent diabetes mellitus, malaria and leishmaniasis^[13,15-17]. In chronic HCV patients, elevated levels of TNF α were detected in liver and serum^[8]. TNF α increases fat deposition in the liver by affecting the hepatic lipogenesis^[18]. Furthermore, Hooper *et al.*^[19] showed that increased levels of TNF α and reduced anti-inflammatory cytokines correlate with the severity of liver injury in non-alcoholic steatohepatitis (NASH) patients.

The role of TNF α -G308A polymorphism in HCV pathogenesis has been examined in different studies, with divergent results^[6,20-22]. These variations may be due to racial background of the studied populations, who have different cytokine gene polymorphisms^[23]. Also, these studies focused on the role of TNF α -G308A in HCV susceptibility and treatment response rate but none of these studies examined the role of TNF α -G308A polymorphism in HCV liver steatosis and fibrosis progression rate. The current study was designed to investigate the association of TNF α -G308A polymorphism with liver inflammation, steatosis and different grades of fibrosis in treatment-naïve Egyptian patients infected with HCV genotype 4.

MATERIALS AND METHODS

Subjects

The study was approved by the Ethics Committee of the National Research Centre, Giza, Egypt according to the Helsinki Declaration of 1975 revised in 2008. A total of 180 subjects, including 120 treatment-naïve HCV infected patients with different fibrosis grades grade who did not receive any treatment and 60 healthy controls, were enrolled in this study. Informed consents were obtained from all study subjects before enrollment in the study and collection of blood samples. The healthy controls (mean age, 42.6 \pm 9.4 years) had normal liver function tests, no history of liver injury and no viral hepatitis, particularly HCV (negative HCV Ab and negative viremia). The control subjects also did not suffer from any other metabolic dysfunctions or bacterial, viral or malignant diseases. The HCV patients (mean age, 43.1 \pm 10.1 years) were admitted to the Endemic Medicine Department of Kasr El Ainy Hospitals at Cairo University. Patients were excluded if they had decompensated cirrhosis, metabolic liver disease, hepatitis B virus, schistosomiasis, alcohol, drug-induced hepatitis or any significant coexisting medical conditions. Laboratory tests, including measurements of alanine aminotransferase (ALT), aspartate transaminase (AST), bilirubin, alkaline phosphatase (ALP), creatinine, complete blood count, body mass index (BMI) and HCV viral load were performed, as well as liver biopsy, for all patients. The extents of liver inflammation, steatosis and different grades of fibrosis were determined according to the METAVIR scoring

system^[24].

Detection of HCV viremia by real time-PCR

Total RNA was extracted from serum using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) to detect the presence of HCV RNA. HCV RNA was detected quantitatively using the Artus HCV RT-PCR Kit (Qiagen) according to the manufacturer's instructions. Genotype of HCV RNA was detected using the INNOLiPA HCV genotyping Kit (Innogenetics, Ghent, Belgium).

Genotyping of TNF α -G308A polymorphism by PCR-restriction fragment length polymorphism analysis

Total DNA was extracted from whole blood of all subjects using the QIAamp Blood Kit (Qiagen) according to the manufacturer's instructions. The TNF α -G308A genotyping was performed using PCR-restriction fragment length polymorphism (RFLP) analysis. The TNF α -308 G/A promoter polymorphism was amplified by PCR using 5'-AGGCAATAGGTTTGTAGGGCCAT-3' as forward primer and 5'-CCTCCCTGCTCCGATTCCG-3' as reverse primer, as previously described by Shmarina *et al.*^[10] and Ho *et al.*^[25]. These primers cover the TNF α -308 polymorphism region and produced a 107 bp PCR fragment. The PCR amplification was carried out in 25 μ L, containing 5-10 μ g DNA, 4 mmol/L MgCl₂ (Promega, Madison, WI, United States), 1 μ mol/L of each primer, 200 μ mol/L dNTPs (Promega), 1 \times PCR buffer (Promega), and 1 U Go Taq DNA polymerase (Promega). The PCR thermal cycling was carried out in an MJ Research cycler instrument. The thermal cycling conditions consisted of initial denaturation at 94 $^{\circ}$ C for 5 min, followed by 35 cycles of denaturation at 94 $^{\circ}$ C for 30 s, annealing at 58 $^{\circ}$ C for 30 s, and extension at 72 $^{\circ}$ C for 1 min, with a final extension at 72 $^{\circ}$ C for 10 min. The PCR fragments were detected by electrophoresis in a 3% agarose gel stained with ethidium bromide.

The G/A allelic polymorphism at TNF α -308 was detected by RFLP analysis. The PCR (107 bp) fragments from all subjects were digested with *Nco*I restriction enzyme. The digestion reaction was carried out in a total volume of 20 μ L, containing 1 \times restriction buffer, 8 μ L PCR product, and 5 U *Nco*I (Promega) according to the manufacturer's recommendations. The *Nco*I restriction digestion was performed at 37 $^{\circ}$ C for 4 h. Afterward, 10 μ L of the digested products were run on a 4% agarose gel stained with ethidium bromide.

Sequence analysis of TNF PCR products

To confirm the results of TNF α -308 PCR-RFLP analysis, some TNF α -308 PCR products were sequenced using the Sanger dideoxynucleotide chain termination method. The TNF α -308 PCR products were purified using the QIAquick PCR Purification Kit (Qiagen). Then, the PCR products were sequenced with the TNF α reverse primer using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems Inc, Carlsbad, CA, United States). After the cycle sequencing reaction, the

Table 1 Clinical data of 120 chronic hepatitis C virus (F0-F4) patients

	HCV patients with different fibrosis grade (n = 120)			
	F0 (n = 30)	F1 (n = 30)	F2-3 (n = 30)	F4 (n = 30)
Age (yr)	38.4 \pm 7.1	41.5 \pm 8.7	43.5 \pm 8.4	48.3 \pm 9.3
Sex (female/male)	13/17	12/18	10/20	9/21
BMI (kg/m ²)	25.5 \pm 2.3	27.6 \pm 3.4	28.7 \pm 2.7	29.5 \pm 2.7
ALT (U/L)	23.4 \pm 7.1	39.8 \pm 23.7	51.1 \pm 20.7	60.1 \pm 20.3
AST (U/L)	22.4 \pm 6.5	34.9 \pm 20.9	48.9 \pm 25.9	58.7 \pm 18.6
ALP (U/L)	113.6 \pm 32.8	122.5 \pm 37.1	158.7 \pm 47.7	153.8 \pm 53.5
Total bilirubin (mg/dL)	0.53 \pm 0.32	0.61 \pm 0.23	0.96 \pm 0.37	1.60 \pm 0.65
Albumin (g/dL)	4.10 \pm 0.33	4.00 \pm 0.38	3.80 \pm 0.45	3.50 \pm 0.39
Platelets ($\times 10^9$ /L)	300.5 \pm 56.2	250.2 \pm 48.4	239.6 \pm 55.6	159.4 \pm 69.5
HCV RNA ($\times 10^3$ IU/mL)	684.3 \pm 124.8	860.1 \pm 1042	1241.5 \pm 1286.7	1501.4 \pm 1661.0
Fibroscan Activity	4.70 \pm 0.75	6.30 \pm 0.36	9.90 \pm 1.78	23.27 \pm 9.30
A0-A1	20 (66.7)	13 (43.3)	10 (33.3)	1 (3.3)
A2-A3	10 (33.3)	17 (56.7)	20 (66.7)	29 (96.7)
Steatosis				
$\leq 33\%$	28 (93.3)	26 (86.7)	16 (53.3)	4 (13.3)
$> 33\%$	2 (6.7)	4 (13.3)	14 (46.7)	26 (86.7)

The clinical data was compared in HCV patients with different fibrosis grades: F0 (n = 30), F1 (n = 30), F2-F3 (n = 30), F4 (n = 30). The quantitative data were represented as mean \pm SD while qualitative data were represented as n (%). HCV: Hepatitis C virus; BMI: Body mass index; ALT: Alanine aminotransferase; AST: Aspartate transaminase; ALP: Alkaline phosphatase.

products were purified using the BigDye XTerminator Purification Kit (Applied Biosystems Inc) and analyzed on the ABI 3500 Genetic Analyzer.

Serum TNF α level measurement

Serum samples were collected and stored at -80 °C. TNF α level in serum was determined in 180 subjects (120 HCV patients and 60 healthy individuals) by TNF enzyme-linked immunosorbent assay (ELISA) kit (Biosource Europe S.A, Nivelles, Belgium) according to the manufacturer's instructions. The concentration of TNF α in serum was determined using a double antibody sandwich ELISA. All samples were analyzed and recombinant standards were included on every plate.

Statistical analysis

The statistical methods of this study were reviewed by a specialized IT development and biomedical statistician. Data were collected, prepared and analyzed using the SPSS software, version 15. Data were expressed as number and percentage for qualitative parameters and as mean \pm SD for quantitative parameters. The frequency of genotypes and alleles in patients and controls were analyzed by χ^2 test. Comparisons of the clinical parameters of different

groups and genotypes were performed by *t* test. Then, stepwise logistic regression analysis was used to identify predictors associated with degree of fibrosis in chronic HCV patients. The OR and 95%CI were calculated to assess the relative risk confidence. *P* value ≤ 0.05 was considered significant, while was considered *P* < 0.01 highly significant.

RESULTS

General characteristics of HCV infected patients

The biochemical, virological and histopathological parameters of 120 HCV-infected patients with different fibrosis grade (F0: n = 30, F1: n = 30, F2-F3: n = 30, F4: n = 30) are summarized in Table 1. There were no significant differences within the different fibrosis groups for age, sex, BMI and HCV RNA viral load. Patients with liver fibrosis grades F2-3 or F4 had statistically significant higher levels of AST, ALT, ALP and total bilirubin and significantly lower platelet count and albumin level, as compared to patients with liver fibrosis grade F0 or F1.

TNF α -308 RFLP and sequence analysis

The amplified TNF α -308 PCR products were digested with *Nco*I restriction enzyme, and run on 4% agarose gel, as shown in Figure 1A. The homozygote AA genotype at TNF α -308 showed the original 107 bp fragment intact (lacking the *Nco*I site), while the homozygote GG genotype showed two fragments of 87 and 20 bp. The heterozygote GA genotype showed three fragments of 87, 20 and 107 bp. Moreover, the sequence results confirmed the integrity of the *Nco*I restriction site's surrounding area and verified the results of the TNF α -308 PCR-RFLP analysis. The sequence results for the TNF α -308 different genotypes are shown in Figure 1B.

Distribution of different TNF α -G308A genotypes in controls and HCV infected patients with different fibrosis grade

Distribution of TNF α -G308A genotypes in HCV infected patients and controls are shown in Table 2. The results showed that the TNF α -G308A SNP frequency was significantly different between controls and HCV infected patients (*P* = 0.001). The TNF α -G308A genotypes in the controls were 66.6% GG, 30% GA and 3.3% AA. The GG genotype in controls (66.6%) is higher than in chronic HCV patients (40%), while the chronic HCV patients had higher GA and AA genotypes (47.5% and 12.5%) compared to controls (30% and 3.3%). Moreover, the G allele is more frequent than the A allele in both controls (81.7% vs 18.3%) and HCV-infected patients (63.8% vs 36.3%), with statistically significant difference (*P* = 0.002). The distribution of TNF α genotypes in HCV patients with different fibrosis grade is shown in Figure 2. The TNF α GG genotype was 60% in F0, 43.3% in F1, 33.3% in

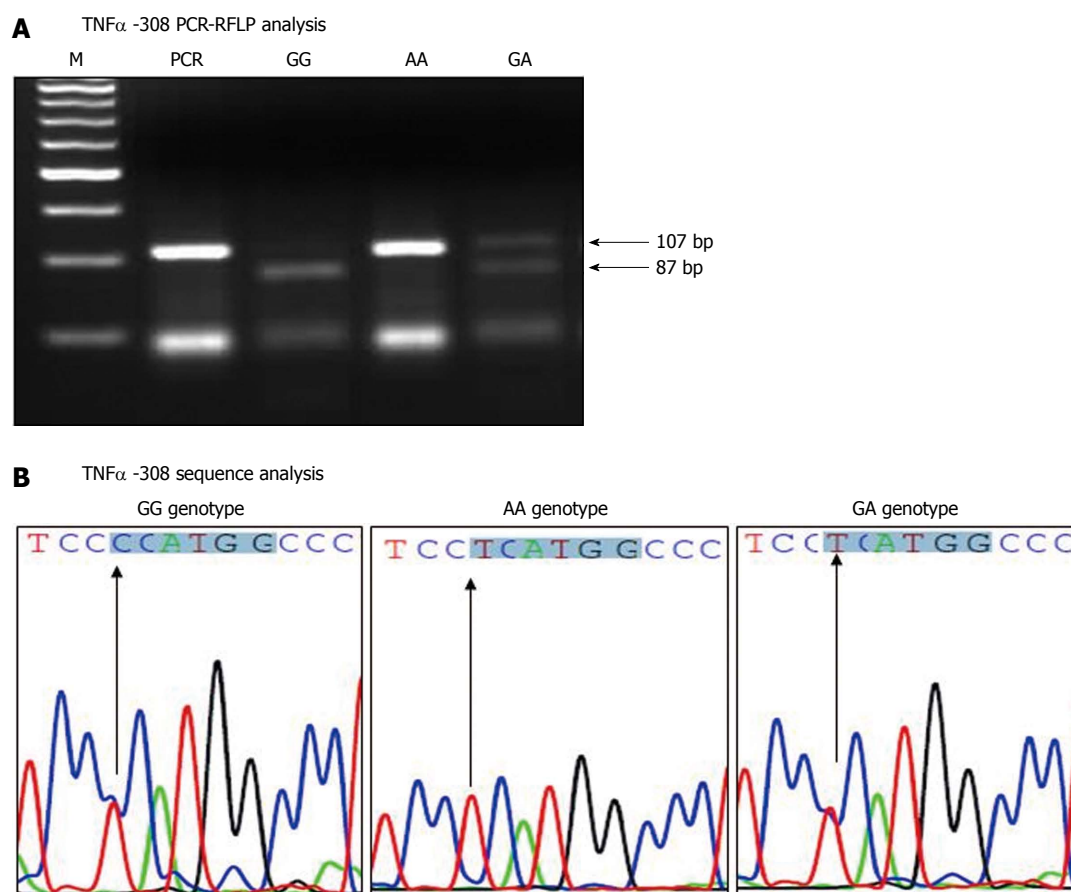


Figure 1 Tumor necrosis factor α -G308A polymorphism analysis. A: TNF α -308 PCR-RFLP analysis, genomic DNA was extracted, amplified by PCR, digested with *NcoI* restriction enzyme and run on a 4% agarose gel. Lanes 1, 2, 3 and 4 correspond to PCR products before digestion, GG homozygote (87 bp), AA homozygote (107 bp) and GA heterozygote (107 and 87 bp) respectively. B: TNF α -308 PCR sequence analysis, the PCR products of different TNF α -308 genotypes were purified and sequenced using the reverse primer. The *NcoI* restriction site is highlighted and the arrow points to the single nucleotide polymorphism. TNF α : Tumor necrosis factor alpha; HCV: Hepatitis C virus; RFLP: Restriction fragment length polymorphism.

Table 2 Distribution of tumor necrosis factor α -G308A polymorphism in controls and hepatitis C virus infected patients with different fibrosis grades (F0-F4) *n* (%)

Polymorphism of TNF α -308	Controls (<i>n</i> = 60)	HCV Patients (<i>n</i> = 120)	F0 patients (<i>n</i> = 30)	F1 patients (<i>n</i> = 30)	F2-3 patients (<i>n</i> = 30)	F4 patients (<i>n</i> = 30)
GG genotype	40 (66.6)	48 (40.0)	18 (60.0)	13 (43.3)	10 (33.3)	7 (23.3)
GA genotype	18 (30.0)	57 (47.5)	10 (33.3)	15 (50.0)	16 (53.4)	16 (53.4)
AA genotype	2 (3.3)	15 (12.5)	2 (6.7)	2 (6.7)	4 (13.3)	7 (23.3)
GA + AA	20 (33.3)	72 (60.0)	12 (40.0)	17 (56.7)	20 (66.6)	23 (76.6)
G allele	98 (81.7)	153 (63.8)	46 (76.7)	41 (68.3)	36 (60.0)	30 (50.0)
A allele	22 (18.3)	87 (36.3)	23 (23.3)	19 (31.7)	24 (40.0)	30 (50.0)

Genotyping of TNF α -G308A was conducted and distribution of different genotypes and alleles was calculated as percentage and compared in 60 controls and 120 chronic HCV patients with different fibrosis grades: F0 (*n* = 30), F1 (*n* = 30), F2-F3 (*n* = 30), F4 (*n* = 30). TNF α : Tumor necrosis factor alpha; HCV: Hepatitis C virus.

F2-F3, and 23.3% in F4 patients, while the (GA + AA) genotypes were 40% in F0, 56.7% in F1, 66.6% in F2-F3, and 76.6% in F4 patients.

Frequency of TNF α -308 genotypes in early and late HCV fibrosis patients

The 120 chronic HCV patients with different fibrosis grade were classified into 60 patients with early fibrosis grade (F0-F1) and 60 patients with late fibrosis

grade (F2-F4). Analysis of the frequency of each TNF α genotype showed that early fibrosis patients have 26.7% of AA, 43.9% of AG and 64.6% of GG genotype, which indicates an increasing trend of having the G allele. The AA genotype was detected in 73.3% of late fibrosis patients vs 26.7% of early fibrosis patients ($P = 0.05$), as shown in Figure 3. In general, the late fibrosis patients had a statistically significant higher rate of A allele than the early fibrosis

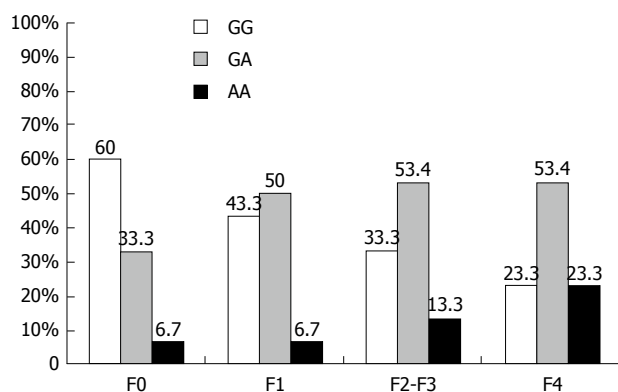


Figure 2 Distribution of tumor necrosis factor α -G308A polymorphism in hepatitis C virus infected patients with different fibrosis grade (F0-F4). Genotyping of TNF α -G308A was conducted for 120 chronic HCV patients (F0-F4) using PCR-RFLP analysis. The different TNF α -G308A genotypes were represented as percentages and compared among HCV patients with different fibrosis grades: F0 ($n = 30$), F1 ($n = 30$), F2-F3 ($n = 30$), F4 ($n = 30$). TNF α : Tumor necrosis factor alpha; HCV: Hepatitis C virus; RFLP: Restriction fragment length polymorphism.

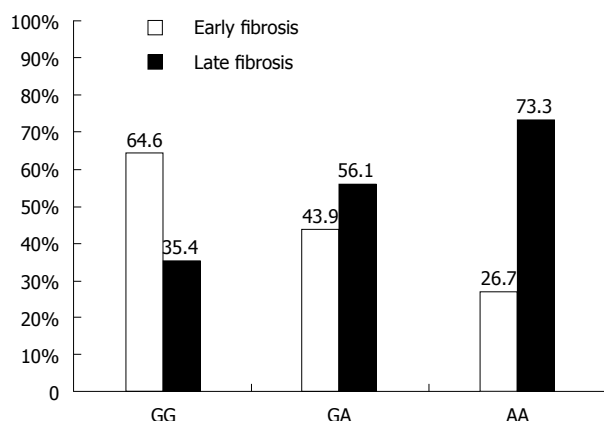


Figure 3 Frequency of each tumor necrosis factor α -308 genotype in early and late hepatitis C virus fibrosis patients. Genotyping of TNF α -G308A was conducted in 120 chronic HCV patients (F0-F4) using PCR-RFLP analysis. The frequency of each genotype (GG, GA, AA) was calculated as percentage and compared in early (F0-F2, $n = 60$) and late (F2-F4, $n = 60$) HCV fibrosis patients. TNF α : Tumor necrosis factor alpha; HCV: Hepatitis C virus; RFLP: Restriction fragment length polymorphism.

patients (62.1% vs 37.9% respectively, $P = 0.04$).

Relation between TNF α genotypes and serum TNF α level in HCV infected patients

The TNF α serum levels were determined in healthy controls and HCV patients with different fibrosis grade. Compared with that in healthy controls, serum TNF α level was gradually elevated in HCV infected patients with different fibrosis grade, as shown in Figure 4. The results showed that the TNF α serum level was 4.01 ± 1.1 pg/mL in controls, 7.9 ± 2.6 pg/mL in F0 patients, 11.3 ± 4.8 pg/mL in F1 patients, 12.9 ± 4.7 pg/mL in F2-F3 patients, and 14.1 ± 4.9 pg/mL in F4 patients. Patients with late fibrosis (F2-F4) had higher TNF levels (13.4 ± 4.8 pg/mL) than patients with early fibrosis (F0-F1) (8.7 ± 2.9 pg/mL). The results showed

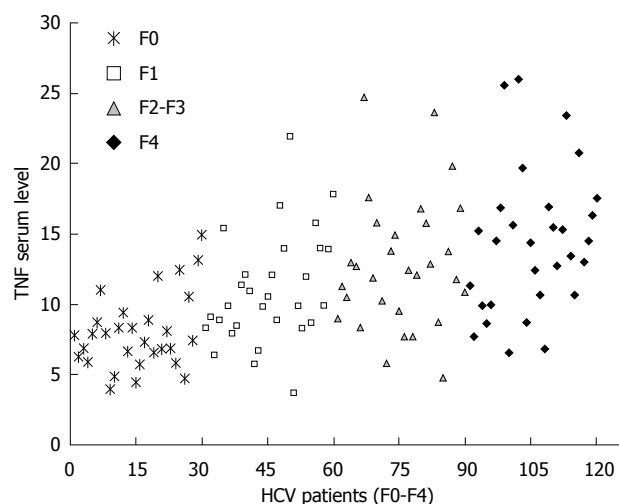


Figure 4 Tumor necrosis factor α serum level in 120 chronic hepatitis C virus patients (F0-F4). The circulating TNF α level was determined using ELISA in 120 chronic HCV patients with different fibrosis grades: F0 ($n = 30$), F1 ($n = 30$), F2-F3 ($n = 30$), F4 ($n = 30$). TNF α : Tumor necrosis factor alpha; HCV: Hepatitis C virus; ELISA: Enzyme-linked immunosorbent assay.

Table 3 Tumor necrosis factor α serum level in 120 hepatitis C virus patients

	TNF α serum levels				<i>P</i> value
	GG	GA	AA	GA + AA	
All fibrosis patients (F0-F4, $n = 120$)	9.1 ± 2.6	12.1 ± 4.9	16.1 ± 4.9	13.1 ± 5.1	0.0001
early fibrosis patients (F0-F1, $n = 60$)	8.3 ± 2.6	10.1 ± 4.5	16.2 ± 4.02	10.9 ± 4.9	0.010
late fibrosis patients (F2-F4, $n = 60$)	10.4 ± 2.1	13.8 ± 4.5	16.1 ± 5.6	14.4 ± 4.9	0.002

The TNF α serum level produced by different TNF α -308 genotypes GG, GA and AA were detected in early and late HCV fibrosis patients. The TNF α serum level is represented as mean \pm SD and was statistically compared in patients with genotype GG to those with AA genotype. $P \leq 0.05$ is considered significant, while $P \leq 0.01$ is highly significant. TNF α : Tumor necrosis factor alpha; HCV: Hepatitis C virus.

that the TNF α serum level was positively correlated to HCV liver fibrosis progression ($P < 0.001$). Moreover, the TNF α serum concentration was significantly higher in patients with A containing genotypes (GA or AA) than in those with GG genotype (10.9 ± 4.9 pg/mL vs 8.3 ± 2.6 pg/mL, $P = 0.01$) in early fibrosis (F0-F1) patients and (14.4 ± 4.9 pg/mL vs 10.4 ± 2.1 pg/mL, $P = 0.002$) in late fibrosis (F2-F4) HCV patients, as shown in Table 3.

Association of TNF α genotypes and different liver pathological grades

The influence of different TNF α genotypes on hepatic parameters and liver pathology (liver inflammation, steatosis, and fibrosis) is shown in Table 4. The results demonstrated that TNF α AA genotype patients have

Table 4 Influence of tumor necrosis factor α -308 genotypes on the liver biochemical and pathological parameters

	TNF genotypes (n = 120)			P value	
	GG (n = 48)	AG (n = 57)	AA (n = 15)	GG vs AA	GG vs GA + AA
AST (U/L)	31.9 \pm 20.6	44.7 \pm 21.6	57.7 \pm 28.7	0.0003	0.0003
ALT (U/L)	34.5 \pm 20.9	46.5 \pm 20.5	62.0 \pm 28.2	0.0001	0.0004
ALP (U/L)	113.7 \pm 36.8	138.3 \pm 41.5	190.1 \pm 46.5	0.0020	0.3500
Albumin (g/dL)	3.9 \pm 0.46	3.9 \pm 0.39	3.6 \pm 0.59	0.0100	0.2000
Total bilirubin (mg/dL)	0.73 \pm 0.41	0.89 \pm 0.51	1.3 \pm 0.68	0.0002	0.0100
Platelets ($\times 10^9$ /L)	254.9 \pm 66.2	224.5 \pm 72.3	205.8 \pm 96.6	0.0360	0.0220
HCV RNA ($\times 10^3$ IU/mL)	862.6 \pm 1295.6	1083.9 \pm 1187.2	1675.5 \pm 1477.6	0.0450	0.2200
Activity					
A0-A1 (n = 44)	25 (56.8)	18 (40.9)	1 (2.3)	0.0290	0.0350
A2-A3 (n = 76)	23 (30.3)	39 (51.3)	14 (18.4)		
Steatosis					
$\leq 33\%$ (n = 74)	36 (48.7)	34 (45.9)	4 (5.4)	0.0280	0.0300
> 33% (n = 46)	12 (26.1)	23 (50.0)	11 (23.9)		
Fibrosis					
F0-F1 (n = 60)	28 (46.7)	30 (50.0)	2 (3.3)	0.0310	0.0400
F2-F4 (n = 60)	20 (33.3)	27 (45.0)	13 (21.6)		

The effect of different TNF α -308 genotypes (GG, GA, AA) on biochemical parameters (ALT, AST, ALP, albumin, total bilirubin), platelets count and pathological parameters (activity, steatosis, fibrosis) was statistically analyzed. The biochemical data were represented as mean \pm SD, while pathological data were represented as n (%). $P \leq 0.05$ is considered significant, while $P \leq 0.01$ is highly significant. TNF α : Tumor necrosis factor alpha; HCV: Hepatitis C virus; ALT: Alanine aminotransferase; AST: Aspartate transaminase; ALP: Alkaline phosphatase.

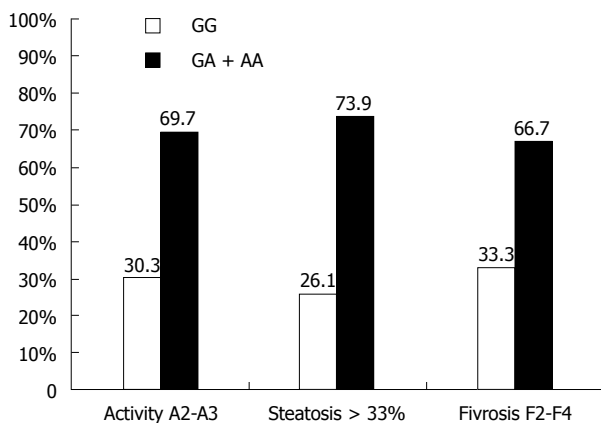


Figure 5 Effect of different tumor necrosis factor α genotypes on liver pathological parameters (inflammation, steatosis and fibrosis). The effect of high expressing TNF α genotypes (GA + AA) were statistically compared with the low expressing TNF α genotype (GG), and the results showed strong effect of (GA + AA) genotypes on liver inflammation ($P = 0.007$), steatosis ($P = 0.005$) and fibrosis ($P = 0.032$). $P \leq 0.05$ is considered significant while $P \leq 0.01$ is highly significant. TNF α : Tumor necrosis factor alpha; HCV: Hepatitis C virus.

significantly higher levels of AST, ALT, ALP and total bilirubin than GG patients ($P = 0.0003$, 0.0001 , 0.002 and 0.0002 respectively). Patients with AA genotype have significantly lower level of albumin and platelets count ($P = 0.01$ and 0.036 respectively). Moreover, the effect of serum TNF α level expressed by genotypes (GA and AA) on liver inflammation, steatosis and fibrosis are shown in Figure 5. The results showed that genotypes GA or AA which express higher TNF serum level were found in 69.7% of high liver inflammation (A2-A3) patients, in 73.9% of severe steatotic patients, and in 66.7% of late fibrotic (F2-F4) patients.

Table 5 Stepwise logistic regression analysis for the effect of tumor necrosis factor α -308 genotypes and alleles on liver disease progression

TNF genotype	Regression coefficient	SE	OR (95%CI)	P value
Activity				
GG vs GA + AA	1.329	0.507	3.776 (1.399-10.194)	0.009
G vs A allele	1.171	0.400	3.226 (1.474-7.060)	0.003
Steatosis				
GG vs GA + AA	1.502	0.580	4.491 (1.441-14.000)	0.010
G vs A allele	1.099	0.373	3.000 (1.445-6.228)	0.003
Fibrosis				
GG vs GA + AA	1.044	0.493	2.841 (1.080-7.472)	0.034
G vs A allele	0.895	0.366	2.446 (1.195-5.008)	0.014

$P \leq 0.05$ is considered significant while $P \leq 0.01$ is highly significant. TNF: Tumor necrosis factor.

Stepwise logistic regression analysis for the effect of TNF α -308 genotypes and alleles on liver disease progression

Different TNF genotypes were investigated to determine their significance in liver disease progression using stepwise logistic regression analysis. The outcome of liver progression was significantly influenced by different TNF genotypes and alleles, as shown in Table 5. Patients with TNF α GA and AA genotypes have increased risk of liver inflammation (A2-A3) (OR = 3.776, 95%CI: 1.399-10.194, $P = 0.009$), severe steatosis (> 33%) (OR = 4.49, 95%CI: 1.441-14.0, $P = 0.010$) and fibrosis progression (F2-F4) (OR = 2.84, 95%CI: 1.080-7.472, $P = 0.034$) than those with GG genotype. In general, patients with A allele have more risk of liver inflammation, steatosis and fibrosis than

those carrying the G allele ($P = 0.003$, 0.003 and 0.014 respectively).

DISCUSSION

Cytokines represent a large family that includes the IFNs, ILs, chemokines and TNF proteins. Cytokines play important roles in the activation and regulation of human immune responses and their production are variable due to SNPs^[26]. Several studies have reported the impact of different cytokines' polymorphisms on the pathogenesis and outcomes of many diseases. Competent cytokine responses to HCV infection are very important to control virus replication, disease progression and treatment response.

TNF α is a diverse multifunctional, proinflammatory and immuno-mediator cytokine. Several studies have shown that the A allele at the TNF α promoter region -308 polymorphism affects the transcriptional level of TNF α gene^[27,28]. There are few data regarding the role of TNF α polymorphism in disease progression and response to antiviral therapy in patients infected with HCV genotype 4. In this study, we examined the association between TNF α -G308A polymorphism and pathological parameters of liver such as inflammation, steatosis and fibrosis in 120 treatment-naïve HCV genotype 4 Egyptian patients.

Current findings showed that TNF α GG genotype is significantly higher in controls than in HCV infected patients (66.6% vs 40%, $P = 0.001$), while the A allele tends to be more frequent in HCV-infected patients than in controls (36.3% vs 18.3%, $P = 0.002$). The (GA + AA) genotypes exhibited gradual increase with progressive fibrosis, with 40% in (F0), 56.7% in (F1), 66.6% in (F2-F3), and 76.6% in (F4). In general, 62.1% of the A allele were found in late fibrosis patients (F2-F4) compared to 37.9% in early fibrosis patients (F0-F1) ($P = 0.015$). Similar results were found by Dogra *et al*^[29], who reported that the frequency of TNF α -308 genotypes was significantly different between healthy controls and HCV patients. The association between TNF α -308A allele and HCV persistence and failure of response to IFN treatment was reported by Radwan *et al*^[30] and Dai *et al*^[31]. Nevertheless, other studies failed to find association between the TNF α -G308A polymorphism and chronicity of HCV infection^[6,20,32].

On the other hand, there were significantly high serum TNF α mean values in late fibrosis (F2-F4) patients, with higher levels in individuals with GA or AA genotypes than those with GG genotype. Similar findings were reported in patients with Crohn's disease, where the TNF α -308A allele was associated with increased TNF α production and inflammatory activity^[33]. Also, Roth-Isigkeit *et al*^[34] reported elevated TNF α serum levels in GA heterozygote individuals undergoing cardiac surgery. Further *in vitro* support of these findings was reported by Abraham and Kroeger^[14], who found that A allele induced mRNA

expression and TNF α production up to 5-fold more than did the G allele.

Several studies have shown that TNF α -308A polymorphism alters TNF α expression levels and changes the immune response. It was reported that TNF α -308A polymorphism can worsen the clinical outcome of many inflammatory and infectious diseases and can increase the risk of some autoimmune diseases. Abraham and Kroeger^[14] defined TNF α as a genetic susceptibility factor for some autoimmune, inflammatory and infectious diseases. High TNF α expression level was also reported to inhibit insulin signaling and decrease adiponectin levels, leading to development of insulin resistance and liver steatosis. Furthermore, it was demonstrated that high circulating TNF α is a potent risk factor for steatosis^[35].

In the present study, we examined the link between high TNF α serum levels and liver inflammation, steatosis and fibrosis. Our results showed that TNF α -308AA genotype patients have significantly higher levels of AST, ALT, ALP and total bilirubin than GG patients. Also, HCV patients with severe liver pathological conditions (inflammation, steatosis and fibrosis) have high frequencies of TNF α (GA or AA) genotypes. These results showed a clear association between severe liver pathological conditions (inflammation, steatosis and fibrosis) and (GA + AA) genotypes ($P = 0.035$, 0.03 and 0.04 respectively). These results suggest that A containing genotypes (GA + AA) express higher TNF α levels than GG genotype and consequently induce inflammatory response that leads to liver inflammation, injury and severe fibrosis^[36].

Our results confirmed the previous studies in which a significant elevation in TNF α serum level was detected in cirrhotic liver patients and was correlated with progression to hepatocellular carcinoma^[22,37,38]. It was reported that TNF α -308AA genotype is associated with the HCV pathogenesis and viral persistence^[21,31]. Aller *et al*^[35] showed that patients with the TNF α mutant genotype (GA or AA) have moderate to severe portal inflammation and fibrosis, contrasting those patients with wild genotype (GG). Also, high TNF α plays a role in fatty liver disease pathogenesis^[35] and leads to severe liver injury in NASH patients^[19]. However, other studies were unable to find any association between TNF α genetic polymorphisms and severity of liver disease or response to antiviral therapy^[6,26,39].

The stepwise logistic regression analysis showed that the TNF α genotypes (GA + AA) were significantly associated with liver inflammation (OR = 3.776, 95%CI: 1.399-10.194, $P = 0.009$), severe steatosis (OR = 4.49, 95%CI: 1.441-14.0, $P = 0.010$) and fibrosis progression (OR = 2.84, 95%CI: 1.080-7.472, $P = 0.034$). The A allele was an independent risk factor for liver inflammation ($P = 0.003$), steatosis ($P = 0.003$) and fibrosis ($P = 0.014$). Therefore, the current results confirm that HCV infected patients carrying one or two A alleles at TNF α -308 have a risk factor for development of severe liver pathological grades.

Several experimental studies showed that the inhibition of TNF α signaling by anti-TNF α antibodies or compounds could reduce inflammation, liver injury and improve fibrosis^[40,41]. However, complete neutralization of TNF α in alcoholic hepatitis patients was associated with serious infectious complications^[42]. Therefore, it was recommended to use pentoxifylline (PTX), which partially attenuates TNF α level and has lower infectious complication rates. It was demonstrated that PTX therapy effectively reduces the liver biochemical parameters and improves the histological injury in NASH patients^[43].

Although liver biopsy is the conventional way to determine the grade of liver fibrosis, it is an invasive method and there is too much sampling. Therefore, there is an increasing need for biomarkers that are specific, sensitive and respond quickly to changes in fibrosis and activity grades. Based on the results of the current study and our previous work on IL28B^[44,45], OAS1 and MxA^[7,46,47], we have presented some potential genetic markers that can be useful in the determination of liver disease progression. In conclusion, the TNF α SNP at nucleotide -308 represents an important genetic marker for developing strategies of prognosis for liver inflammation, steatosis and fibrosis in HCV infected patients.

COMMENTS

Background

Hepatitis C virus (HCV) infection is highly endemic in Egypt and the mortality rate of HCV-associated liver diseases reaches 67%. Both viral and host factors play important roles in controlling HCV liver disease progression and response to treatment. Host genetic factors and immunological response to HCV infection can affect the pathogenesis of liver diseases. The single nucleotide polymorphisms (SNPs) are responsible for inter-individual differences in cytokines' and cellular antiviral proteins' production and secretion. Tumor necrosis factor alpha (TNF α) is a proinflammatory and antiviral cytokine that plays a major role in liver injury and initiation of the fibrosis process. The TNF α expression is considered a key molecular link between liver inflammation, steatosis, and fibrosis. TNF α transcription level is affected by the TNF α -G308A polymorphism. In Egypt, more than 90% of patients are infected with HCV genotype 4. Therefore, we designed the current study to investigate the association of the TNF α -G308A polymorphism with liver pathological changes in treatment-naïve chronic HCV genotype 4 patients with different fibrosis grades (F0-F4).

Research frontiers

Chronic hepatitis C infection can cause severe liver diseases, such as fibrosis, cirrhosis, liver failure and hepatocellular carcinoma. Liver fibrosis and cirrhosis could not be reversed but the liver scarring can be improved with HCV treatment. However, timing and rules for HCV genotype 4 treatment without major complications and relapse are under investigation by research studies and clinical trials. The research hotspot is currently how to determine and understand which HCV patients are likely to have chronic infection, progressive liver disease or to not respond significantly to HCV treatment. Therefore, there is a high need for a prognostic model that contains many genetic factors for staging and prediction of HCV liver disease progression, representing an approach that guides therapeutic interventions and prevents further spread of liver fibrosis and hepatic failure.

Innovations and breakthroughs

The TNF α SNP at nucleotide -308 represents an important genetic marker

to predict the liver disease progression rate in treatment-naïve chronic HCV genotype 4 infected patients. There is a demanding need for biological and genetic markers' application for making a decision as to whether the HCV patient will have progressive liver disease or not. These data clearly improve the predication by detecting the TNF α -308 polymorphism and assessing the TNF α serum level. The data presented in this study demonstrated that the TNF α expression level provide a predictive value for liver disease progression rate.

Applications

The data of this study will help hepatologists and gastroenterologists to better predict liver disease progression in chronic HCV infected patients, to make better decisions on liver disease treatment, and to design novel therapeutic interventions that will control HCV-associated liver disease complications.

Terminology

SNPs in HCV infected patients lead to different immune responses, which significantly influence the progression of chronic HCV infection and response to therapy. TNF α is a diverse multifunctional, proinflammatory and immunomediator cytokine. The A allele at the TNF α promoter region -308 polymorphism affects the transcriptional level of the TNF α gene. High TNF α level in HCV infected patients may accelerate liver disease progression and complications.

Peer-review

This study added new information regarding a subtyping (4) of HCV and its relation with the TNF α cytokine.

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Basic Study

Effects of Lizhong Tang on gastrointestinal motility in mice

Min Cheol Lee, Wooram Ha, Jinhyeong Park, Junghoon Kim, Yunjin Jung, Byung Joo Kim

Min Cheol Lee, Byung Joo Kim, Division of Longevity and Biofunctional Medicine, Healthy Aging Korean Medical Research Center, School of Korean Medicine, Pusan National University, Yangsan 50612, South Korea

Wooram Ha, Jinhyeong Park, Junghoon Kim, Division of Pharmacology, School of Korean Medicine, Pusan National University, Yangsan 50612, South Korea

Yunjin Jung, College of Pharmacy, Pusan National University, Busan 46241, South Korea

Author contributions: Lee MC and Kim BJ designed the research; Lee MC, Ha W, Park J and Kim J performed the experiments; Jung Y and Kim BJ analyzed the data; and Lee MC and Kim BJ wrote the paper.

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Correspondence to: Byung Joo Kim, PhD, Associate Professor,

Division of Longevity and Biofunctional Medicine, School of Korean Medicine, Pusan National University, Beomeori, Mulgeum-eup, Yangsan, Gyeongsangnamdo, 50612, South Korea. vision@pusan.ac.kr
Telephone: +82-51-5108469
Fax: +82-51-5108420

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Abstract

AIM

To investigate the effects of Lizhong Tang, a traditional Chinese medicine formula, on gastrointestinal motility in mice.

METHODS

The *in vivo* effects of Lizhong Tang on GI motility were investigated by measuring the intestinal transit rates (ITRs) and gastric emptying (GE) values in normal mice and in mice with experimentally induced GI motility dysfunction (GMD).

RESULTS

In normal ICR mice, the ITR and GE values were significantly and dose-dependently increased by Lizhong Tang (ITR values: 54.4% \pm 1.9% vs 65.2% \pm 1.8%, P < 0.01 with 0.1 g/kg Lizhong Tang and 54.4% \pm 1.9% vs 83.8% \pm 1.9%, P < 0.01 with 1 g/kg Lizhong Tang; GE values: 60.7% \pm 1.9% vs 66.8% \pm 2.1%, P < 0.05 with 0.1 g/kg Lizhong Tang and 60.7% \pm 1.9% vs 72.5% \pm 1.7%, P < 0.01 with 1 g/kg Lizhong Tang). The ITRs of the GMD mice were significantly reduced compared with those of the normal mice, which were significantly and dose-dependently reversed by Lizhong Tang. Additionally, in loperamide- and cisplatin-induced

models of GE delay, Lizhong Tang administration reversed the GE deficits.

CONCLUSION

These results suggest that Lizhong Tang may be a novel candidate for development as a prokinetic treatment for the GI tract.

Key words: Lizhong Tang; Gastrointestinal disorders; Motility; Intestinal transit rate; Gastric emptying

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Core tip: Lizhong Tang, a traditional Chinese medicinal formula, has been widely used in China, Japan, and South Korea for many years to ameliorate gastrointestinal (GI) disorders. Our data suggest that Lizhong Tang is a novel candidate for development as a prokinetic agent for treatment of GI motility dysfunctions in man.

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INTRODUCTION

Lizhong Tang, also known as Yijung-tang or Richu-to, is a traditional Chinese medicine (TCM) formula^[1] and is composed of Radix Ginseng (*Panax ginseng* C.A. Meyer), Rhizoma Zingiberis (*Zingiber officinale* Roscoe), Rhizoma Atractylodis Macrocephalae (*Atractylodes macrocephala* Koidz.), and Radix Glycyrrhizae (*Glycyrrhiza uralensis* Fisch)^[2]. Lizhong Tang is widely used in traditional medicine to treat gastrointestinal (GI) disorders, such as vomiting, diarrhea, stomach pain, chronic gastritis, stomach bleeding, and GI ulceration, in China, Japan, and South Korea^[1-3]. However, no studies have been conducted to evaluate the effect of Lizhong Tang on GI motility.

Prokinetic agents are medications that enhance coordinated GI motility and the transit of content in the GI tract mainly by amplifying and coordinating the GI muscular contractions. In addition, prokinetic therapy should be considered as a means to improve gastric emptying and symptoms of gastroparesis, balancing the benefits and risks of treatment^[4]. Recently, prokinetic therapy has been shown to improve the symptoms and quality of life in patients with GI motility disorders^[5]. Therefore, there has been an increasing need to develop safer and more effective gastroprokinetic agents.

In our previous report, we investigated the effects of Lizhong Tang on mouse small intestine interstitial cells of Cajal (ICC)^[6]. These cells are the pacemaker

cells of GI muscles and generate rhythmic oscillations in membrane potentials known as slow waves^[7-9] by activating Ca²⁺ entry through L-type Ca²⁺ channels in smooth muscles to initiate GI contractions^[10,11]. In this report, we found that Lizhong Tang affected GI motility by modulating pacemaker activity in ICC through internal Ca²⁺- and phospholipase (PLC)-dependent pathways^[6]. However, despite the widespread use of Lizhong Tang to treat GI disorders, little is known about its regulatory effects on GI motility. Therefore, we performed this study to investigate the effects of Lizhong Tang on the mouse GI tract *in vivo*.

MATERIALS AND METHODS

Preparation of the standard solutions and sample extracts

Liquiritin, 6-gingerol, ononin, glycyrrhizin, ginsenoside Rg1, isoliquiritin, and atractylenolide III were accurately weighed and dissolved in methanol (all at 100 µg/mL) to prepare standard solutions. Lizhong Tang powder was dissolved in the water and then filtered through a 0.2 µm syringe filter (BioFACTTM, South Korea) prior to injection in the high performance liquid chromatography (HPLC).

Chromatographic conditions

An Agilent 1200 (Agilent Technologies, Palo Alto, CA, United States) equipped with an autosampler, degasser, quaternary solvent pump, and diode array detector (DAD) was used for the analysis. The data were acquired using ChemStation software (Agilent Technologies, Palo Alto, CA, United States). Separation was performed on a Capcell Pak Mg II C₁₈ column (4.6 mm × 250 mm, 5 µm; Shiseido, Tokyo, Japan) at 35 °C. The mobile phase consisted of water containing 0.1% trifluoroacetic acid (A) and acetonitrile (B), and gradient elution was conducted as follows: 5% (B) for 0-1 min; 5%-10% (B) for 1-5 min and held for 5 min; 10%-15% (B) for 10-12 min and held for 4 min; 15%-20% (B) for 16-18 min and held for 4 min; 20%-23% (B) for 22-25 min and held for 6 min; 23%-28% (B) for 31-32 min and held for 8 min; and 28%-65% (B) for 40-80 min and held for 2 min. The column was then re-equilibrated using 5% (B) for the subsequent analyses. The flow rate was set at 1.0 mL/min, and the detection wavelengths were 205, 230, 250, 280, and 360 nm.

Animals

Male ICR mice (Samtako BioKorea Co., Ltd., Osan, South Korea) weighing 23-30 g were used to investigate the effects of the Lizhong Tang extract on the GI tract *in vivo*. The animals were maintained under controlled conditions (21 °C ± 3 °C, relative humidity 50% ± 6%, lights on 6 a.m.-6 p.m.). The mice were allowed free access to a commercial diet and tap water, but were fasted for 24 h before the

experiments. All experiments were conducted between 10 a.m. and 6 p.m.

Measurement of intestinal transit rate using Evans blue staining

We used Evans blue solution [5%, w/v, in distilled water (DW)] to determine the intestinal transit rates (ITR) of the Lizhong Tang extract *in vivo*. The Evans blue solution was administered (0.1 mL/kg of body weight; i.g.) through an orogastric tube 30 min after the Lizhong Tang extract was intragastrically (i.g.) administered to the normal ICR mice. The animals were sacrificed 30 min after Evans blue administration, and the intestinal transit distances of the dye were determined by measuring the distance the Evans blue dye had migrated in the intestine from the pylorus to its most distal point. Intestinal transit was quantified using the ITRs (%), which were calculated by expressing the distance the Evans blue dye traveled in 30 min as a percentage of the total small intestine length (from the pylorus to the ileal terminus).

Induction of GI motility dysfunction in mice

Two experimental GI motility dysfunction models were used: an acetic acid (AA)-induced peritoneal irritation mouse model and a STZ-induced diabetic mouse model. For the AA model, peritoneal irritation was induced by administering AA to ICR mice 30 min after the i.g. administration of the Lizhong Tang extract (or DW as vehicle) by intraperitoneally (i.p.) injecting 10 mL/kg AA (0.6%, w/v, in saline) as previously described^[12-14]. After injecting AA, the mice were placed in individual cages and allowed to recover for 30 min. Male ICR mice (aged 5 wk) were used for the STZ-induced diabetic mouse model. The mice were randomly allocated to two groups: a control group or a diabetic group. The mice were fasted overnight and an STZ (Sigma-Aldrich, St. Louis, MO, United States) solution was administered i.p. on the following day to produce diabetes. Fresh STZ was prepared in 0.1 mol/L ice-cold citrate buffer (pH 4.0) and administered at 200 mg/kg body weight^[15]. The control mice were i.p. administered the same volume of 0.1 mol/L citrate buffer. The animals had free access to food and water and were maintained under standard conditions (24-27 °C, RH 60%-65%) under a 12 h light/dark cycle. Two months after the STZ injection, blood was withdrawn from a tail vein after an 8 h fast and the blood glucose concentrations were measured using a ONE-TOUCH Select Simple kit (Johnson and Johnson Medical Company). Diabetes was defined as a blood glucose level of > 16 mmol/L. No mortality occurred during the study period, and no mouse recovered from STZ-induced diabetes.

Evaluation of gastric emptying

As previously described by Scarpignato *et al.*^[16], the mice were fasted for 24 h with free access to water^[14].

Gastric emptying (GE) was performed by administering a 0.05% (w/v) phenol red solution (0.5 mL/mouse) 30 min after treatment with the Lizhong Tang extract. Twenty min later, the mice were sacrificed and the stomachs were immediately removed, cut into several pieces, placed into 5 mL of 0.01 N NaOH, and homogenized. The homogenates were treated with 0.2 mL of 20% trichloroacetic acid per mL of homogenate. The mixtures were centrifuged for 10 min at 1050 × *g*, and the supernatants (0.05 mL) were added to 0.5 N NaOH (0.2 mL). The absorbances of these mixtures were measured using a spectrometer at 560 nm. The GE value (%) was calculated as $100 - (A/B) \times 100$, where A is the test stomach absorbance (560 nm) and B is the control stomach absorbance (560 nm) immediately after phenol red administration.

Drugs

All drugs were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, United States). In addition, an aqueous extract of the dried immature fruit of *Poncirus trifoliata* Raf. (PF) was prepared as previously described^[17,18] and its prokinetic activities were compared with the Lizhong Tang extract. PF is one of the most popular traditional folk medicines used in South Korea and is obtained from Rutaceae fruits. PF has been shown to possess unique, potent prokinetic activities in normal rodents and rodents with GI motility dysfunction (GMD)^[13,17].

Statistical analysis

The results are expressed as the means ± SE. Statistical analysis was performed using Student's *t* test or analysis of variance followed by Tukey's multiple comparison test, as appropriate. Statistical significance was accepted for *P* values < 0.05.

RESULTS

Identification of standard compounds in the Lizhong Tang extract

The following components of the Lizhong Tang extract were detected by HPLC using commercial standards (retention time): liquiritin (25.1 min); ononin (35.9 min); isoliquiritin (36.2 min); ginsenoside Rg1 (36.7 min); glycyrrhizin (58.2 min); 6-gingerol (63.0 min); and atractylenolide III (65.8 min) (Figure 1).

Effects of the Lizhong Tang extract on ITR in normal mice

After 30 min, the mean ITR (%) for Evans blue in normal mice was 54.4% ± 1.9% (Figure 2). PF (1 g/kg), which has been shown to have prokinetic activity in the GI tract^[17,18], significantly accelerated the ITR [79.4% ± 2.3% (*P* < 0.01)], similar to the Lizhong Tang extract, which dose-dependently increased the ITR (%) [ITR values at 0.01, 0.1 and 1 g/kg were 56.1% ± 2.1%, 65.2% ± 1.8% (*P* < 0.01) and

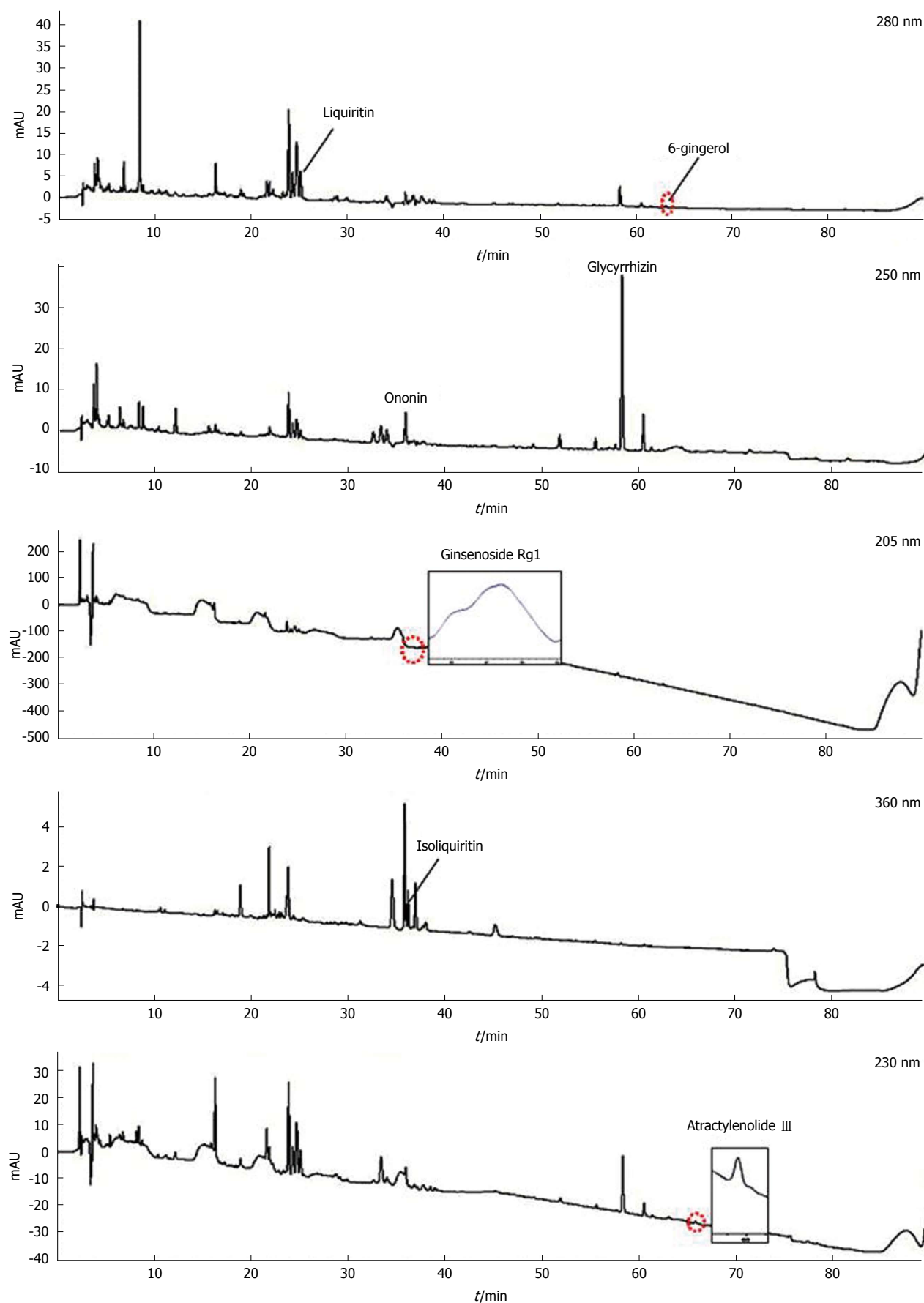


Figure 1 Chromatograms of liquiritin, 6-gingerol, ononin, glycyrrhizin, ginsenoside Rg1, isoliquiritin, and atractylenolide III in Lizhong Tang extract.

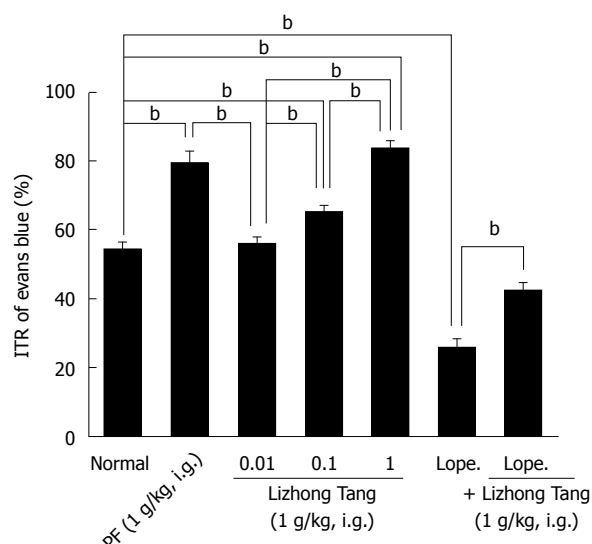


Figure 2 Effects of the Lizhong Tang extract on the intestinal transit rates (%) in normal mice. Intestinal transit rates (ITRs) (%) values of normal mice that were pretreated with the Lizhong Tang extract prior to Evans blue administration ($n = 15$ for each bar). The bars represent mean values \pm SE. ^b $P < 0.01$; significantly different from the normal controls. PF: *Poncirus trifoliata* Raf; Lope.: Loperamide.

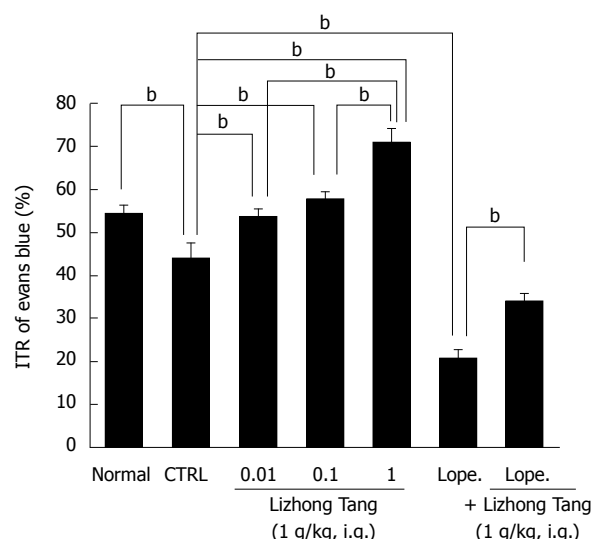


Figure 4 Effects of the Lizhong Tang extract on the Intestinal transit rates (%) values in STZ-induced diabetic mice. Intestinal transit rates (ITRs) (%) values of the STZ mice induced 2 mo before the i.g. administration of Evans blue ($n = 12$ per bar). The bars represent mean values \pm SE. ^b $P < 0.01$; significantly different from the STZ-induced diabetic controls. CTRL: Control; Lope.: Loperamide.

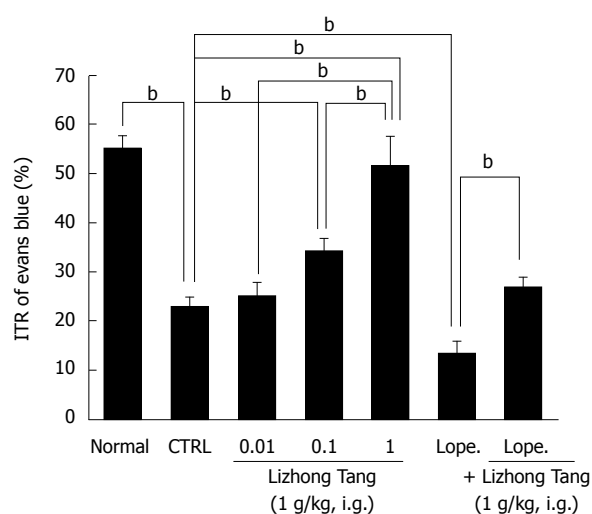


Figure 3 Effects of the Lizhong Tang extract on the intestinal transit rates (%) in AA mice. Intestinal transit rates (ITRs) (%) values of AA mice induced 30 min before the i.g. administration of Evans blue ($n = 12$ per bar). The bars represent mean values \pm SE. ^b $P < 0.01$; significantly different from the AA controls. CTRL: Control; Lope.: Loperamide.

$83.8\% \pm 1.9\%$ ($P < 0.01$), respectively; Figure 2]. Loperamide decreased the ITR (%), which is consistent with previous reports^[19], and the Lizhong Tang extract inhibited this loperamide-induced decrease in ITR [ITR value for loperamide was $56.1\% \pm 2.1\%$; and ITR value for loperamide with the Lizhong Tang extract was $65.2\% \pm 1.8\%$ ($P < 0.01$); Figure 2].

Effects of the Lizhong Tang extract on ITR in mice with GMD

We used the AA and STZ-induced diabetic mouse

models of experimental GMD to examine the effect of the Lizhong Tang extract on GI motility. As mentioned above, the AA mouse model showed a significant retardation of ITR (%) [$23.2\% \pm 1.5\%$ ($P < 0.01$ vs normal); Figure 3]. However, a significant inhibition of this retardation was observed when the mice were intragastrically administered 0.01, 0.1, or 1 g/kg of the Lizhong Tang extract [$25.3\% \pm 2.4\%$, $34.5\% \pm 2.1\%$ ($P < 0.01$) and $51.8\% \pm 5.7\%$ ($P < 0.01$), respectively; Figure 3]. No abnormal clinical signs or changes were observed in the AA mice after administration of the Lizhong Tang extract. In addition, loperamide decreased the ITR (%) in the AA mice [$13.5\% \pm 2.4\%$ ($P < 0.01$)], and the Lizhong Tang extract increased this value [$26.7\% \pm 2.1\%$ ($P < 0.01$); Figure 3]. Furthermore, the STZ-induced diabetic mice also showed a significant ITR (%) retardation ($44.1\% \pm 3.5\%$; Figure 4), which was also significantly inhibited by treatment with the Lizhong Tang extract at 0.01, 0.1 or 1 g/kg [$53.8\% \pm 1.5\%$ ($P < 0.01$), $57.7\% \pm 1.4\%$ ($P < 0.01$) and $71.5\% \pm 3.0\%$ ($P < 0.01$), respectively; Figure 4]. No abnormal clinical signs or changes were observed in the STZ-induced diabetic mice after the administration of the Lizhong Tang extract. In addition, loperamide decreased the ITR in the STZ-induced diabetic mice [$20.6\% \pm 1.8\%$ ($P < 0.01$)], and the Lizhong Tang extract increased this value [$40.6\% \pm 2.2\%$ ($P < 0.01$); Figure 4]. These results indicate that the Lizhong Tang extract increased the ITR in mice with GMD.

Effect of the Lizhong Tang extract on accelerating GE

In normal mice, the groups treated with the

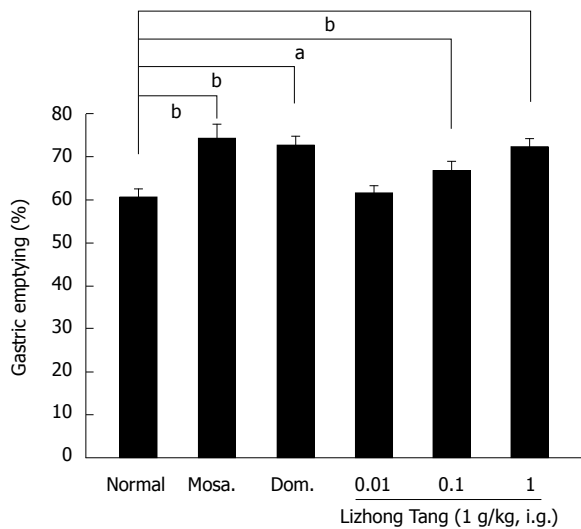


Figure 5 Effect of the Lzhong Tang extract on accelerating gastric emptying. After a 24 h fast, the animals ($n = 7$ /each group) were orally administered the indicated dosages of the Lzhong Tang extract, 5 mg/kg of a 5-HT₄ receptor agonist (mosapride), 5 mg/kg of a dopamine receptor antagonist (domperidone), or distilled water (DW; controls). The gastric emptying (GE) percentages were calculated as described in the Materials and Methods. The bars represent means \pm SE. ^a $P < 0.05$; ^b $P < 0.01$; significantly different from the normal controls. Mosa.: Mosapride. Dom.: Domperidone.

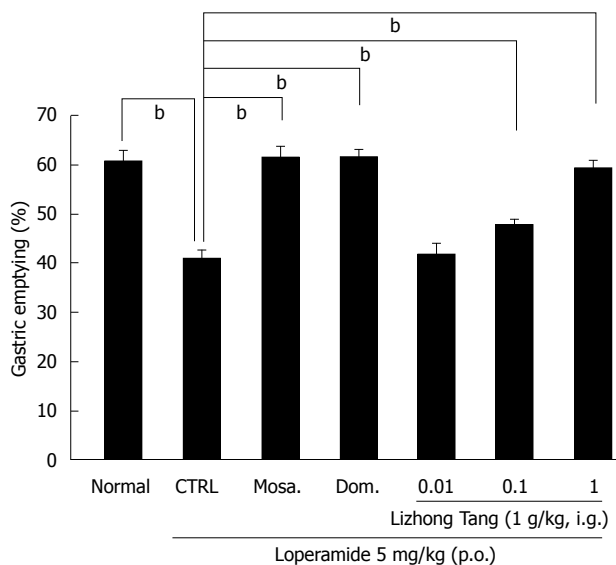


Figure 6 Lzhong Tang extract ameliorates loperamide-induced delays in gastric emptying. After a 24 h fast, the animals ($n = 6$ /each group) were orally administered the indicated dosages of the Lzhong Tang extract, 5 mg/kg of a 5-HT₄ receptor agonist (mosapride), 5 mg/kg of a dopamine receptor antagonist (domperidone), or distilled water (DW; control). The loperamide-induced gastric emptying (GE) delay was inhibited by the Lzhong Tang extract. The GE percentages were calculated as described in the Materials and Methods. The bars represent means \pm SE. ^b $P < 0.01$; significantly different from the normal controls. Mosa: Mosapride; Dom: Domperidone; p.o.: per os.

Lzhong Tang extract (0.01, 0.1 and 1 g/kg) showed significantly enhanced GE (%) values compared to that of the normal group [the GE values with 0.01, 0.1 and 1 g/kg of the Lzhong Tang extract were $61.7\% \pm 1.6\%$, $66.8\% \pm 2.1\%$ ($P < 0.05$) and $72.5\% \pm 1.7\%$ (P

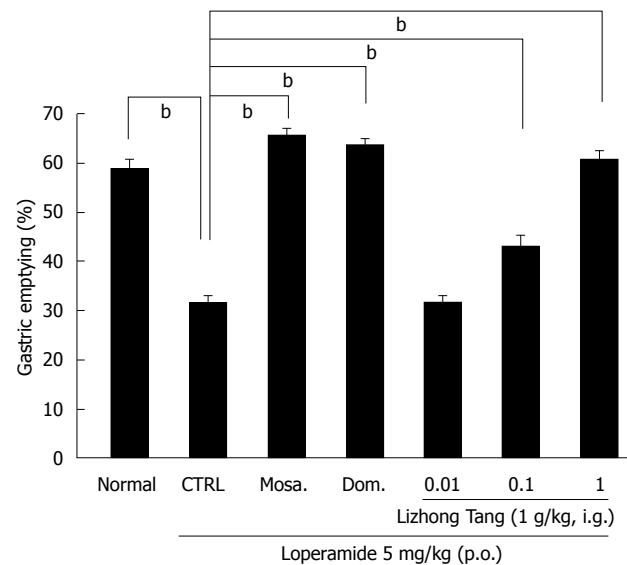


Figure 7 Lzhong Tang extract ameliorated cisplatin-induced delays in gastric emptying. After a 24 h fast, the animals ($n = 6$ /each group) were orally administered the indicated dosages of the Lzhong Tang extract, 5 mg/kg of a 5-HT₄ receptor agonist (mosapride), 5 mg/kg of a dopamine receptor antagonist (domperidone), or distilled water (DW; control). The Lzhong Tang pretreatment prevented the cisplatin-induced gastric emptying (GE) delay. The GE percentages were calculated as described in the Materials and Methods. The bars represent mean values \pm SE. ^b $P < 0.01$; significantly different from the normal controls. Mosa: Mosapride; Dom: Domperidone; i.p.: Intraperitoneally.

< 0.01), respectively; Figure 5]. Its effects were dose-dependent in the dosage range from 0.01 g/kg to 1 g/kg, and 1 g/kg of the Lzhong Tang extract displayed effects similar to those of 5 mg/kg mosapride [$74.4\% \pm 3.3\%$ ($P < 0.01$)] and 5 mg/kg domperidone [$72.9\% \pm 1.9\%$ ($P < 0.01$)] (Figure 5). Next, we examined loperamide-induced and cisplatin-induced models of GE delay to determine whether the Lzhong Tang extract could increase GE in abnormally depressed GE models. In the loperamide-induced model of GE delay, the GE value was lower than normal [$40.9\% \pm 1.6\%$ ($P < 0.01$); Figure 6], and this decrease was recovered by treatment with the Lzhong Tang extract at doses from 0.01 to 1 g/kg [the GE values for the Lzhong Tang extract at 0.01, 0.1 and 1 g/kg were $41.8\% \pm 2.2\%$, $47.8\% \pm 1.2\%$ ($P < 0.01$) and $59.4\% \pm 1.5\%$ ($P < 0.01$), respectively; Figure 6]. The maximal effect was obtained at 1 g/kg, and at this dose, the effect of the Lzhong Tang extract was comparable to that of 5 mg/kg mosapride [$61.4\% \pm 2.3\%$ ($P < 0.01$)] or 5 mg/kg domperidone [$61.5\% \pm 1.7\%$ ($P < 0.01$)] (Figure 6). In addition, in the cisplatin-induced model of GE delay, the decreased GE was recovered by treatment with the Lzhong Tang extract (0.01, 0.1 and 1 g/kg) [GE values of the Lzhong Tang extract at 0.01, 0.1 and 1 g/kg were $31.7\% \pm 1.3\%$, $43.1\% \pm 2.1\%$ ($P < 0.01$) and $60.8\% \pm 1.7\%$ ($P < 0.01$), respectively; Figure 7]. The maximal effect was obtained at 1 g/kg, and at this level, the effect of the Lzhong Tang extract was comparable to that of 5 mg/kg mosapride [65.6%

$\pm 1.4\%$ ($P < 0.01$)] or 5 mg/kg domperidone [63.7% $\pm 1.2\%$ ($P < 0.01$)] (Figure 7).

DISCUSSION

GI motility results from the coordinated contractions of the *tunica muscularis*, which forms the outer wall of the alimentary canal from the distal esophagus to the external anal sphincter^[20]. The pathogenesis of primary intestinal motility disorders is probably multifactorial and includes structural and biochemical abnormalities, forms of intestinal pseudo-obstruction, and mucosal inflammation^[21].

The GI tract exhibits spontaneous mechanical contractions and electrical pacemaker potentials^[7,8], and these pacemaker potentials are the basic determinant of GI smooth muscle activity^[7,8]. Recent studies have shown that ICC act as the pacemakers and conductors of electrical slow waves in the GI tract^[7,8].

In our previous report, we investigated the effects of Lizhong Tang on ICC in the GI tract^[6]. Lizhong Tang induced pacemaker potential depolarizations that were mediated by non-selective cationic channels, intracellular calcium release, and PLC-dependent pathways. Therefore, we suggested that Lizhong Tang might have gastroprokinetic effects on ICC^[6]. In addition, many scientists have studied the effects of Lizhong Tang. For example, Lizhong Tang has been shown to have therapeutic effects, such as immunomodulatory, anticancer, antitoxic, and antioxidant effects, and to modulate gastric acid secretion^[22-24]. Furthermore, in traditional medicine, it is used to treat the spleen or kidney deficiencies associated with the common symptoms of many diseases, such as pasty loose stools, heavy menstrual bleeding, soreness, and weakness of the lower back and knees^[2,25,26]. Moreover, Lizhong Tang possesses potent anti-osteoporotic activity and has been suggested for use in the treatment of postmenopausal osteoporosis^[3]. In addition, Lizhong Tang protects the gastric mucosa from acute ethanol-induced gastric injury and has been suggested as a treatment for acute gastric injury^[1]. However, despite the considerable use of Lizhong Tang, little is known about its regulatory effects on GI motility *in vivo*.

In this study, the Lizhong Tang extract significantly and dose-dependently accelerated ITR (Figure 2). In experimental GMD (AA mouse and STZ-induced diabetic mouse) models, the Lizhong Tang extract significantly inhibited GMD-induced retardation (Figures 3 and 4), and Lizhong Tang extract-treated mice had significantly greater GE values than normal mice; 1 g/kg of the Lizhong Tang extract displayed effects similar to those of mosapride and domperidone (Figure 5). Furthermore, in abnormally depressed (loperamide- and cisplatin-induced) GE models, the Lizhong Tang extract increased GE (Figures 6 and 7).

Prokinetic drugs, which enhance GI motor function by acting on a variety of neurotransmitter receptors, have been used to treat patients with GI

motility disorders^[27] and are regarded as one of the most efficacious therapeutics for this disorder^[28,29]. Cholinergic agonists, the original promotility agents, stimulated muscarinic M₂-type receptors on the smooth muscle cells, but their effectiveness in motility disorders is inconsistent^[30]. Metoclopramide and domperidone, dopamine antagonists, have been the most widely used as prokinetic agents^[31], but their long-term use has been complicated by a trend toward tolerance and a significant incidence of central nervous system (CNS) side effects^[32]. Cisapride was shown to promote esophageal peristalsis, augment lower esophageal sphincter pressure, and accelerate gastric emptying^[33]. However, the use of this drug is now restricted due to serious cardiac arrhythmias related to a prolonged QT interval^[34]. Mosapride, a selective 5-HT₄ agonist, is available as a prokinetic agent in a number of Asian countries, but the efficacy data are contradictory^[35]. Itopride is a dopamine D₂ antagonist with prokinetic effects that is devoid of CNS or cardiovascular side effects and causes minimal elevations of prolactin levels^[36]. In this study, we did not directly compare the GE and intestine motility rates with these prokinetics agents. However, in a previous study, we showed that Lizhong Tang depolarized the pacemaker potentials through G-protein-, PLC- and Ca²⁺-dependent pathways. Moreover, the nonselective cationic cation channel was involved in these effects^[6]. Therefore, we believe that Lizhong Tang might mimic the major excitatory neurotransmitters of the GI tract and act as a gastroprokinetic agent. Additionally, herbal products may be an attractive alternative based on the perception of their "natural" approach and their low risk of side effects^[37]. Therefore, we believe that Lizhong Tang may be a good gastroprokinetic agent, and in the future, we should compare the experimental results with those of known prokinetics and analyze the side effects.

In summary, in normal ICR mice, both the ITR and GE values were significantly and dose-dependently increased by treatment with the Lizhong Tang extract. Furthermore, the ITRs of GMD mice were significantly reduced compared with those of the normal mice, and these reductions were significantly and dose-dependently reversed by treatment with the Lizhong Tang extract. In addition, in the loperamide-induced and cisplatin-induced model of GE delay, the Lizhong Tang extract prevented the observed GE delays. Taken together, our results suggest that Lizhong Tang is a good candidate for the development of a gastroprokinetic agent.

COMMENTS

Background

Lizhong Tang [composed of Radix Ginseng (*Panax ginseng* C.A. Meyer), Rhizoma Zingiberis (*Zingiber officinale* Roscoe), Rhizoma Atractylodis Macrocephalae (*Atractylodes macrocephala* Koidz.) and Radix Glycyrrhizae (*Glycyrrhiza uralensis* Fisch.)] is a traditional Chinese medicinal formula that

has been widely used in China, Japan and South Korea for many years to ameliorate the symptoms of gastrointestinal (GI) disorders. However, despite the considerable use of Lzhong Tang in traditional medicine to treat GI dysfunction, little was known of its regulatory effects on GI motility *in vivo*.

Research frontiers

Lzhong Tang is a good candidate for development as a gastroprokinetic agent.

Innovations and breakthroughs

In normal ICR mice, both the ITRs and GE values were significantly and dose-dependently increased by treatment with Lzhong Tang (0.1-1 g/kg). The ITRs of the GMD mice were significantly reduced compared with those of the normal mice, and the values were significantly and dose-dependently reversed by treatment with Lzhong Tang (0.1-1 g/kg). Moreover, in loperamide-induced and cisplatin-induced models of GE delay, Lzhong Tang prevented the observed GE delays.

Applications

Lzhong Tang may be a new target or a novel candidate prokinetic agent for the pharmacological treatment of GI motility disorders.

Peer-review

This study is relevant, interesting, is written in suitable English, and has a correct methodological design. It is important to emphasize the contribution that this study provides for integration between western and eastern medicine, which is fundamental to the advancement of modern science.

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Retrospective Study

¹⁸F-fluorodeoxyglucose positron emission tomography/ computed tomography comparison of gastric lymphoma and gastric carcinoma

Xiao-Feng Li, Qiang Fu, You-Wen Dong, Jian-Jing Liu, Xiu-Yu Song, Dong Dai, Cong Zuo, Wen-Gui Xu

Xiao-Feng Li, Qiang Fu, You-Wen Dong, Jian-Jing Liu, Xiu-Yu Song, Dong Dai, Cong Zuo, Wen-Gui Xu, Department of Molecular Imaging and Nuclear Medicine, Tianjin Medical University Cancer Institute and Hospital, Tianjin 300060, China

Xiao-Feng Li, Qiang Fu, You-Wen Dong, Jian-Jing Liu, Xiu-Yu Song, Dong Dai, Cong Zuo, Wen-Gui Xu, National Clinical Research Center for Cancer, Key Laboratory of Cancer Prevention and Therapy, Tianjin 300060, China

Author contributions: Li XF designed the study, analyzed the data and wrote the paper; Fu Q, Dong YW and Zuo C collected the data; Liu JJ, Song XY and Dai D performed the data analysis; Xu WG designed the study, and revised the paper; all authors have read and approved the final version to be published.

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Correspondence to: Wen-Gui Xu, MD, Professor, Department of Molecular Imaging and Nuclear Medicine, Tianjin Medical University Cancer Institute and Hospital, Huan-Hu-Xi Road, Ti-Yuan-Bei, Hexi District, Tianjin 300060, China. w Xu06@tmu.edu.cn
Telephone: +86-22-23340123-5923
Fax: +86-22-23537796

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Abstract

AIM

To compare ¹⁸F-fluorodeoxyglucose positron emission tomography/computed tomography (¹⁸F-FDG PET/CT) features in gastric lymphoma and gastric carcinoma.

METHODS

Patients with newly diagnosed gastric lymphoma or gastric carcinoma who underwent ¹⁸F-FDG PET/CT prior to treatment were included in this study. We reviewed and analyzed the PET/CT features of gastric wall lesions, including FDG avidity, pattern (focal/diffuse), and intensity [maximal standard uptake value: (SUVmax)]. The correlation of SUVmax with gastric

clinicopathological variables was investigated by χ^2 test, and receiver-operating characteristic (ROC) curve analysis was performed to determine the differential diagnostic value of SUVmax-associated parameters in gastric lymphoma and gastric carcinoma.

RESULTS

Fifty-two patients with gastric lymphoma and 73 with gastric carcinoma were included in this study. Abnormal gastric FDG accumulation was found in 49 patients (94.23%) with gastric lymphoma and 65 patients (89.04%) with gastric carcinoma. Gastric lymphoma patients predominantly presented with type I and type II lesions, whereas gastric carcinoma patients mainly had type III lesions. The SUVmax (13.39 ± 9.24 vs 8.35 ± 5.80 , $P < 0.001$) and SUVmax/THKmax (maximal thickness) (7.96 ± 4.02 vs 4.88 ± 3.32 , $P < 0.001$) were both higher in patients with gastric lymphoma compared with gastric carcinoma. ROC curve analysis suggested a better performance of SUVmax/THKmax in the evaluation of gastric lesions between gastric lymphoma and gastric carcinoma in comparison with that of SUVmax alone.

CONCLUSION

PET/CT features differ between gastric lymphoma and carcinoma, which can improve PET/CT evaluation of gastric wall lesions and help differentiate gastric lymphoma from gastric carcinoma.

Key words: Gastric lymphomas; Gastric carcinomas; ^{18}F -fluorodeoxyglucose positron emission tomography/computed tomography; Maximal standard uptake value; Maximal thickness; Differential diagnosis

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Core tip: ^{18}F -fluorodeoxyglucose positron emission tomography/computed tomography (PET/CT) feature in gastric lymphomas compared to that in gastric carcinomas were investigated. Gastric lymphoma patients predominantly presented with type I and type II lesions, whereas gastric carcinoma patients mainly with type III lesions. The SUVmax and SUVmax/THKmax were both higher in patients with gastric lymphomas compared to that in patients with gastric carcinomas. A ROC curve analysis suggested a better performance of SUVmax/THKmax in the evaluation of gastric lesions in comparison with that of SUVmax alone. The differences existed in the PET/CT feature could improve the PET/CT evaluation of gastric lesions and contribute to the identification of gastric lymphomas from gastric carcinomas.

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INTRODUCTION

Lymphoma, mainly non-Hodgkin's lymphoma (NHL), may be extranodal in origin in 25%-40% of patients, depending on geography^[1-3]. The gastrointestinal tract is the most common site of primary extranodal NHL, occurring in 4%-20% of patients^[4], accounting for 20%-30% of extranodal cases at diagnosis^[5]. Second only to gastric carcinoma, gastric lymphoma is an important malignant tumor of the stomach. Histopathology of gastric lymphoma is predominantly high-grade diffuse large B-cell lymphoma (DLBCL) and low-grade mucosa-associated lymphoid tissue (MALT) lymphoma^[3].

Imaging plays an important role in noninvasive evaluation of patients with extranodal lymphoma before treatment^[6,7]. Hybrid positron emission tomography (PET)/computed tomography (CT) integrates ^{18}F -fluorodeoxyglucose (FDG) PET and CT scanning, thus simultaneously providing functional, metabolic information based on PET and structural, anatomical information based on CT^[8-10]. A growing number of studies have supported the application of ^{18}F -FDG PET/CT in initial staging, treatment response assessment, and follow-up of patients with gastric lymphoma of various histological subtypes^[11-14]. However, differences were also present in some previous studies, due in part to lower FDG accumulation in low-grade lymphoma than in aggressive lymphoma, and to the presence of physiological tracer uptake in the stomach^[12,15].

Endoscopic examination and direct biopsy are well-established methods for differential diagnosis between gastric lymphoma and gastric carcinoma^[16-23]. However, ^{18}F -FDG PET/CT has the advantages of detecting gastric lymphoma that is limited to the submucosal stage, which may be missed by gastroscopy, and in finding unanticipated lesions outside the stomach^[23,24]. In addition, ^{18}F -FDG PET/CT is significant for the diagnosis of gastric lesions in patients for whom endoscopic examination is not acceptable.

Clinical manifestations and radiological features of gastric lymphoma and gastric carcinoma are in general nonspecific, such as abdominal pain, dyspepsia, gastric ulcers, and irregular thickness of the gastric wall. Besides, the marked differences between gastric lymphoma and gastric carcinoma with regard to therapeutic options and prognosis further highlight the significance of accurate detection and differentiation of the two tumors. To the best of our knowledge, few studies have focused on the imaging differences between gastric lymphoma and gastric carcinoma using ^{18}F -FDG PET/CT^[18,19]. The purpose of the present investigation was to characterize the PET/CT performance in evaluation of gastric lymphoma in comparison with that in gastric carcinoma.

Table 1 Characteristics of patients with gastric lymphomas and gastric carcinomas *n* (%)

Variables	Gastric lymphomas	Gastric carcinomas	<i>P</i> value
Total number of patients	52	73	
Age, yr, median (range)	56 (8-90)	62 (31-84)	0.026 ¹
Gender (male/female)	29/23	48/25	0.258 ²
Histopathological subtype, <i>n</i>			
	DLBCL: 33 MALT: 19	Mucinous: 13 Non-mucinous: 60	
Stage, <i>n</i>	Lugano (I / II 1/ II 2/IV) 19/7/2/24	TNM (I / II / III/IV) 12/18/12/31	0.326 ³
Involved regions			
Cardia	7 (13.5)	30 (41.1)	0.012 ²
Fundus	16 (30.8)	14 (19.2)	0.245 ²
Body	40 (76.9)	32 (43.8)	0.059 ²
Antrum	28 (53.8)	26 (35.6)	0.205 ²
≥ 2 regions	34 (65.38)	20 (27.40)	0.009 ²
THKmax, cm, mean (range)	1.97 (0.3-6.6)	2.00 (0.3-9.2)	0.913 ⁴
Splenomegalia	12 (23.1)	6 (8.2)	0.046 ²
Involved lymph nodes in retroperitoneal space below renal hilus	15 (28.8)	8 (11.0)	0.037 ²
Mucosal ulceration	18 (34.62)	53 (72.60)	0.023 ²

¹The value was compared by Wilcoxon's rank-sum test; ²The value was compared by Pearson χ^2 test; ³The value was compared by Pearson Kruskal-Wallis ANOVA test; ⁴The value was compared by Student's *t*-test. DLBCL: Diffuse large B cell lymphoma; MALT: Mucosa associated lymphoid tissue; THKmax: The maximal thickness.

MATERIALS AND METHODS

Patients

Consecutive patients with newly diagnosed gastric lymphomas and with newly diagnosed gastric carcinomas by ¹⁸F-FDG PET/CT performed about one week prior to any treatment, and underwent operation between July 2014 and January 2006 in our institution were included in this study. All diagnoses were confirmed by endoscopic biopsy or postoperative pathological findings. This study was reviewed and approved by the Tianjin Medical University Cancer Institute and Hospital Institutional Review Board, and written informed consent was obtained from all the patients. Patients were staged according to the Lugano classification and the Tumor Node Metastasis (TNM) classification, respectively^[8]. The demographic and clinicopathological characteristics of the included patients are presented in Table 1. It is worth mentioning that gastric carcinoma patients were divided into subgroups of mucinous adenocarcinoma and non-mucinous adenocarcinoma to facilitate the interpretation of the ¹⁸F-FDG PET/CT results, and mucinous adenocarcinoma subgroup consisted of gastric mucinous adenocarcinoma and signet ring cell carcinoma.

¹⁸F-FDG PET/CT imaging

The patients were required to fast at least 6 h prior to ¹⁸F-FDG PET/CT examination, with injection of approximately 3.70-4.81 MBq/kg. Blood glucose was measured to ensure the level was below 6.8 mmol/L. After injection, patients were kept lying comfortably for an uptake period of 45-60 min. Before the examination, patients were asked to drink 500-800 mL water to distend the stomach and to accelerate renal tracer elimination. Scanning from head to thigh was performed using a PET/CT system (Discovery ST4, General Electric Healthcare, Waukesha, WI, United States). The protocol included an initial CT scan followed by PET data acquisition. The initial CT was performed with 120 kV, 100 mA and a slice thickness of 5 mm. PET data were obtained in a three-dimensional mode with an acquisition time of 2 min for each bed position (for a total of 6-8 bed positions). The CT-based attenuation-corrected PET images were reconstructed using an iterative algorithm. After completion of data acquisition, separate PET images, CT images and fused PET/CT data were available for review in coronal, sagittal and axial planes using the manufacturer's review station (Xeleris, General Electric Healthcare).

¹⁸F-FDG PET/CT interpretation

The ¹⁸F-FDG PET/CT images were visually interpreted by a consensus of at least two experienced nuclear medicine physicians who were aware of the clinical manifestation, but blinded to the specific histological diagnosis of the patients.

The maximal thickening measurement of the gastric wall based on CT component was recorded to define the size of the lesion. To determine the intensity of gastric FDG uptake semi-quantitatively, the maximal standard uptake value (SUVmax) was measured. The patterns of PET/CT scan were classified into three subtypes according to the infiltrative and thickening extent of the lesion in the stomach: type I, infiltrating more than one-third of the total gastric wall and with diffuse thickening; type II, infiltrating less than one-third and with segmental thickening; and type III, with local uptake and local thickening.

Statistical analysis

The data were expressed as mean \pm SD. Student's *t* test, Wilcoxon's rank-sum test, analysis of variance and χ^2 test were used to determine the statistical difference in demographic and clinical characteristics, SUVmax and categorical data between patients with various histological subtypes. The relationship between SUVmax and gastric clinicopathological variables was investigated by χ^2 test. A receiver-operating characteristic (ROC) curve analysis was performed to identify the values of SUVmax or SUVmax/maximal thickness (THKmax) in the differential diagnosis of

Table 2 Incidence, pattern and intensity of gastric ^{18}F -FDG uptake in patients with gastric lymphomas and gastric carcinomas *n* (%)

	Gastric lymphoma	Gastric carcinoma	<i>P</i> value
Presence of gastric FDG uptake	52 (100)	72 (98.63)	0.957 ¹
Gastric FDG uptake > liver	49 (94.23)	65 (89.04)	0.829 ¹
PET/CT pattern			
Type I	23 (44.23)	9 (12.33)	0.002 ¹
Type II	22 (42.31)	14 (19.18)	0.038 ¹
Type III	7 (13.46)	50 (68.49)	< 0.001 ¹
SUVmax (mean \pm SD)	13.39 \pm 9.24	8.35 \pm 5.80	< 0.001 ²
SUVmax/THKmax (mean \pm SD)	7.96 \pm 4.02	4.88 \pm 3.32	< 0.001 ²

¹The value was compared by Pearson χ^2 test; ²The value was compared by Student's *t* test. FDG: Fluorodeoxyglucose; PET/CT: Positron emission tomography/computed tomography; SUVmax: Maximal standard uptake value; THKmax: The maximal thickness.

gastric lymphomas and gastric carcinomas, and the total area under the curve (AUC), 95%CI and a best cutoff threshold of SUVmax or SUVmax/THKmax were calculated to quantify the differential diagnostic value of these indicators. The statistical methods of this study were reviewed by Yu-Bei Huang from the Department of Epidemiology and Biostatistics in our hospital.

All calculation and statistical analyses were performed using the statistical package for social science 21.0 version (SPSS, iNC, Chicago, IL, United States). *P* < 0.05 was considered statistically significant.

RESULTS

Patient characteristics

Fifty-two patients with gastric lymphoma and 73 with gastric carcinoma were included in this study. As expected, the histopathological subtype of gastric lymphoma was predominantly high-grade DLBCL (*n* = 33) or low-grade MALT lymphoma (*n* = 19). Gastric carcinoma patients were divided into mucinous adenocarcinoma (*n* = 13) or non-mucinous adenocarcinoma (*n* = 60) subgroups to facilitate comparison with gastric lymphoma patients. The incidence of the involved regions of the stomach (the cardia, fundus, body and the antrum) was 13.5% (7/52), 30.8% (16/52), 76.9% (40/52) and 53.8% (28/52) for gastric lymphoma and 41.1% (30/73), 19.2% (14/73), 43.8% (32/73) and 35.6% (26/73) for gastric carcinoma, respectively. The THKmax of the gastric wall lesions in patients with gastric lymphoma and gastric carcinoma is compared in Table 1. There was no significant difference with regard to THKmax in patients with gastric lymphoma in comparison with gastric carcinoma (*P* = 0.913). Twelve cases (23.1%) with splenomegaly and 15 (28.8%) with retroperi-

toneal lymph node involvement below the renal hilus were observed in the gastric lymphoma group, while the corresponding numbers in the gastric carcinoma group were lower (*P* = 0.046, *P* = 0.037). Unlike gastric carcinoma, which is an epithelium-derived malignancy, gastric lymphoma is derived from the submucous layer and mostly infiltrates beneath the mucinous membrane, so mucosal ulceration was less common than in gastric carcinoma (34.62% vs 72.6%, *P* = 0.023) (Table 1).

^{18}F -FDG uptake in gastric lesions

The presence of gastric ^{18}F -FDG uptake and SUVmax are summarized in Table 2. Gastric FDG uptake was demonstrated in all 52 patients with gastric lymphoma and in 72 of the 73 patients (98.63%) with gastric carcinoma (*P* = 0.957). However, abnormal gastric FDG accumulation was deemed present if the intensity of gastric ^{18}F -FDG uptake was higher than the hepatic uptake. Forty-nine (94.23%) gastric lymphoma patients and 65 (89.04%) gastric carcinoma patients had increased gastric FDG uptake. The SUVmax was higher in patients with gastric lymphoma compared with gastric carcinoma (13.39 \pm 9.24 vs 8.35 \pm 5.80, *P* < 0.001). With regard to the ^{18}F -FDG PET/CT pattern of gastric wall lesions, the incidence of type I lesions (Figure 1) (*P* = 0.002) and type II lesions (Figure 2) (*P* = 0.038) was significantly higher, but the incidence of type III lesions (Figure 3) (*P* < 0.001) was significantly lower in gastric lymphoma than gastric carcinoma patients. Fu *et al.*^[19] suggested SUVmax/THKmax as a valid and practical biomarker in discriminating primary gastric lymphoma from advanced gastric carcinoma. As illustrated in Table 2, SUVmax/THKmax was significantly higher in patients with gastric lymphoma in comparison with gastric carcinoma (7.96 \pm 4.02 vs 4.88 \pm 3.32, *P* < 0.001).

Association of SUVmax with clinicopathological features

SUVmax was higher in gastric lymphoma patients with DLBCL than in those with MALT (18.41 \pm 7.78 vs 4.66 \pm 2.72, *P* < 0.001) and higher in patients with advanced Lugano stage (II 1/II 2/IV) than in those with stage I (15.53 \pm 8.87 vs 9.97 \pm 8.88, *P* = 0.026) (Table 3). In gastric carcinoma patients, SUVmax was higher in the non-mucinous adenocarcinoma subgroup than in mucinous adenocarcinoma subgroup (9.02 \pm 6.14 vs 5.28 \pm 2.06, *P* = 0.032) and higher in advanced TNM stage (III/IV) than in stage I/II gastric carcinoma patients (10.57 \pm 6.27 vs 5.17 \pm 2.96, *P* < 0.001) (Table 4). With regard to THKmax, there were no significant differences between patients with gastric lymphoma and gastric carcinoma according to cell types and TNM/Lugano stage. We divided patients into low SUVmax (< mean value) and high SUVmax (\geq mean value) subgroups. Association of SUVmax with clinicopathological features was evaluated by χ^2 test among patients with gastric lymphoma and

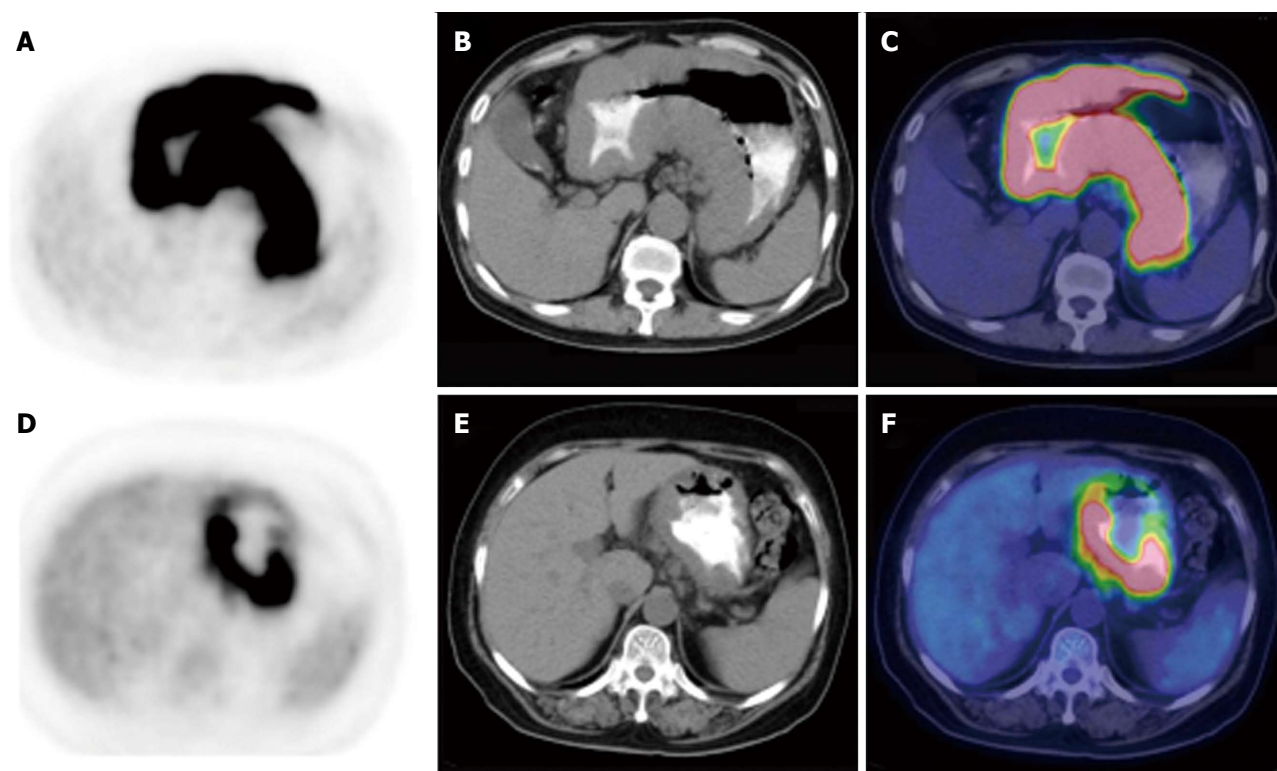


Figure 1 Comparison of gastric lymphoma and gastric carcinoma with diffuse fluorodeoxyglucose uptake. A-C: PET (left column), CT (middle column) and PET/CT fused images (right column) of a 74-year-old man with DLBCL (SUVmax 24.5, THKmax 4.3 cm); D-F: A 64-year-old woman with poorly differentiated gastric adenocarcinoma (SUVmax 28.2, THKmax 2.4 cm). CT: Computed tomography; PET: Positron emission tomography; SUVmax: Maximal standard uptake value; THKmax: Maximal thickness.

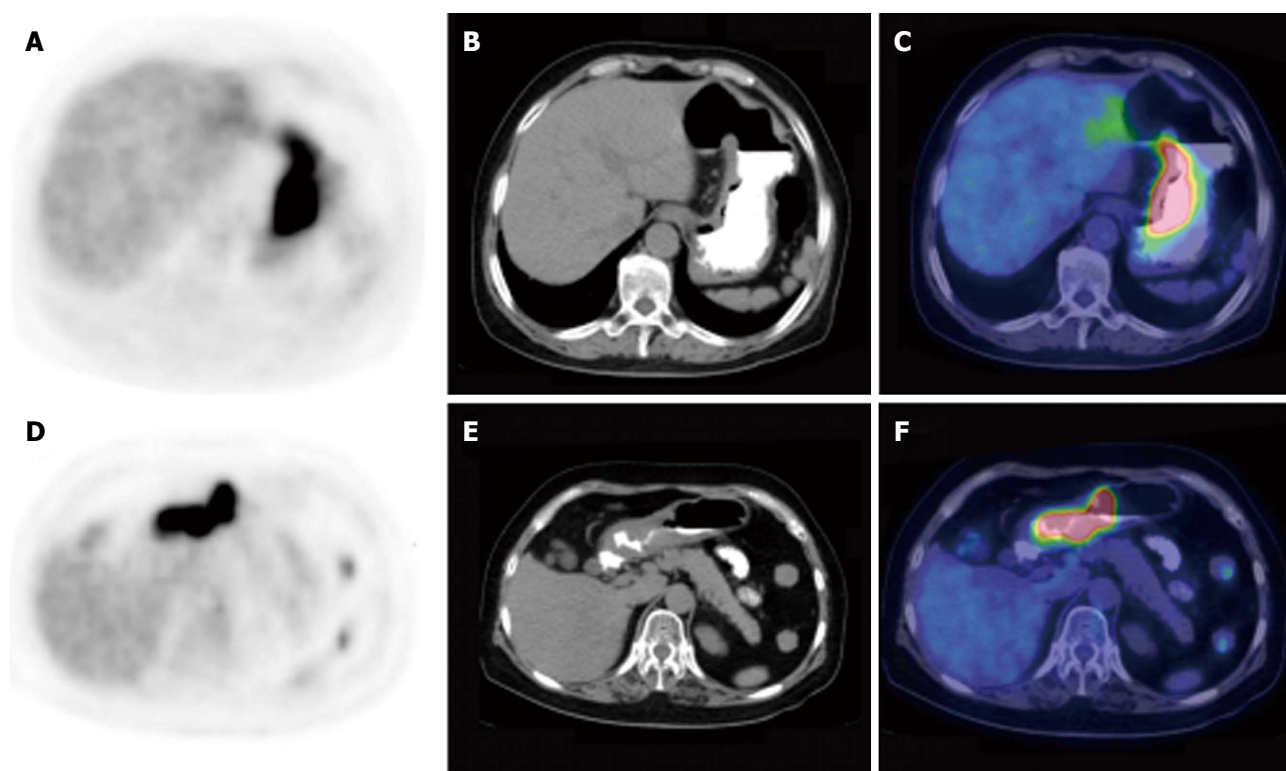


Figure 2 Comparison of gastric lymphoma and gastric carcinoma with segmental fluorodeoxyglucose uptake. A-C: PET (left column), CT (middle column) and PET/CT fused images (right column) of a 58-year-old woman with DLBCL (SUVmax 27.4, THKmax 1.9 cm); D-F: A 69-year-old woman with gastric tubular adenocarcinoma (SUVmax 17.1, THKmax 1.0 cm). CT: Computed tomography; PET: Positron emission tomography; SUVmax: Maximal standard uptake value; THKmax: Maximal thickness.

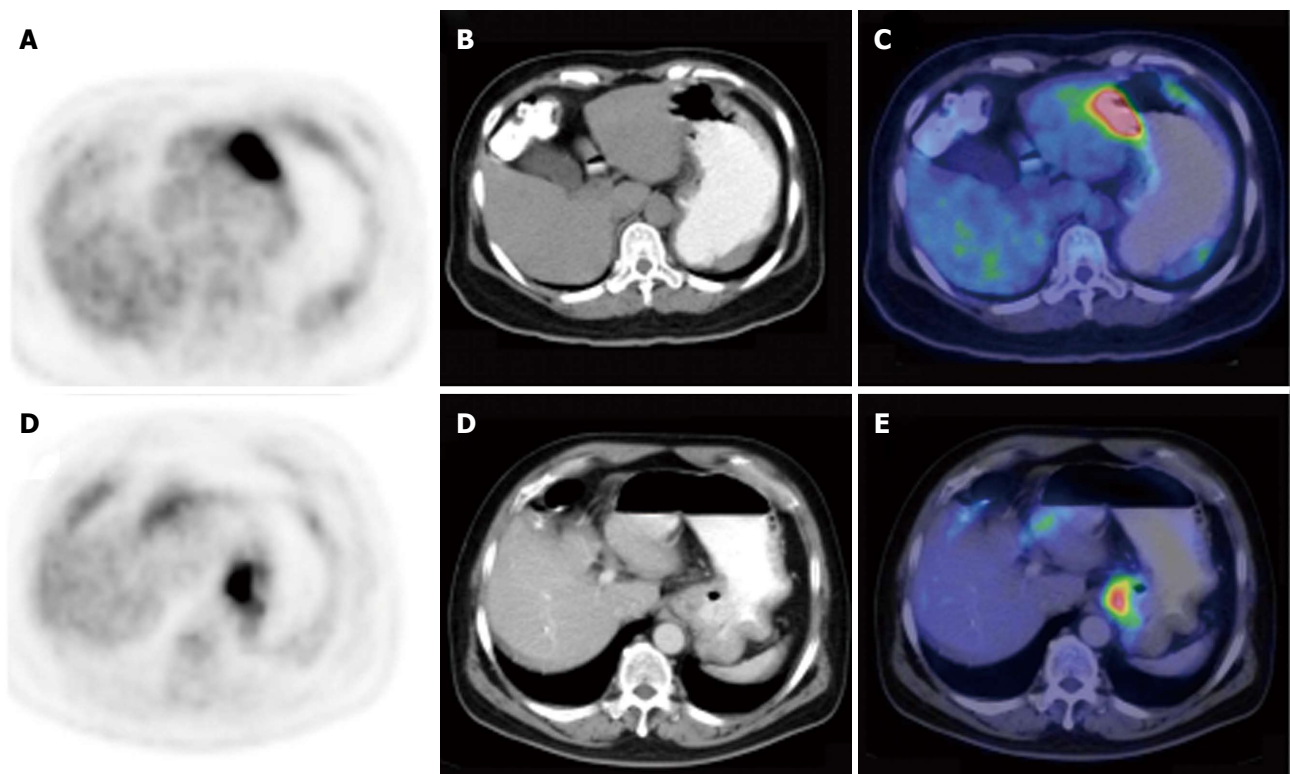


Figure 3 Comparison of gastric lymphoma and gastric carcinoma with local fluorodeoxyglucose uptake. A-C: PET (left column), CT (middle column) and PET/CT fused images (right column) of a 54-year-old woman with DLBCL (SUVmax 13.4, THKmax 2.0 cm); D-F: A 60 year-old man with poorly differentiated gastric adenocarcinoma (SUVmax 9.1, THKmax 1.9 cm) CT: Computed tomography; PET: Positron emission tomography; SUVmax: Maximal standard uptake value; THKmax: Maximal thickness.

Table 3 Association of SUVmax with clinicopathological features among patients with gastric lymphomas						
Characteristics	n	SUVmax and THKmax		SUVmax		
		mean ± SD	P value	High (n)	Low (n)	P value
Sex						
Male	29	14.31 ± 9.58	0.423 ¹	14	15	0.510 ²
Female	23	12.22 ± 8.85		9	14	
Age (yr)						
< mean	22	12.23 ± 8.25	0.446 ¹	10	12	0.879 ²
≥ mean	30	14.23 ± 9.95		13	17	
Histopathological subtype						
DLBCL	33	18.41 ± 7.78 2.33 ± 1.43 ⁴	< 0.001 ³	22	11	< 0.001 ⁵
MALT	19	4.66 ± 2.72 1.36 ± 1.25 ⁴		1	18	
Lugano stage						
I	19	9.97 ± 8.88 1.52 ± 1.19 ⁴	0.026 ¹	6	13	0.270 ²
II 1/ II 2/IV	33	15.53 ± 8.87 2.23 ± 1.51 ⁴		17	16	

¹The value was compared by Student's *t* test; ²The relation with variables was evaluated by Pearson χ^2 test; ³The value was compared by Wilcoxon's rank-sum test; ⁴The value of THKmax according to the cell-types and Lugano staging; ⁵The relation with variables was evaluated by continuity correction χ^2 test. SUVmax: Maximal standard uptake value; THKmax: The maximal thickness.

gastric carcinoma. As shown in Tables 3 and 4, Pearson correlation analysis suggested a significant association of high-grade gastric lymphoma (DLBCL) ($P < 0.001$), non-mucinous adenocarcinoma ($P = 0.046$) and advanced stage gastric carcinoma ($P < 0.001$) with high SUVmax. However, investigation of the relationship of SUVmax to advanced stage gastric

lymphoma did not identify a strong positive correlation.

Comparative value of SUVmax and SUVmax/THKmax in differential diagnosis of gastric lymphoma and gastric carcinoma

Comparative receiver-operating characteristic (ROC) curves were generated to obtain the best cutoff thre-

Table 4 Association of SUVmax with clinicopathological features among patients with gastric carcinomas

Characteristics	n	SUVmax and THKmax		SUVmax		
		mean \pm SD	P value ¹	High (n)	Low (n)	P value ²
Sex						
Male	48	7.55 \pm 4.53	0.457 ²	15	33	0.280 ³
Female	25	9.89 \pm 7.54		11	14	
Age (yr)						
< mean	35	7.14 \pm 4.56	0.088 ¹	9	26	0.090 ³
\geq mean	38	9.46 \pm 6.61		17	21	
Histopathological subtype						
Mucinous	13	5.28 \pm 2.06	0.032 ²	1	12	0.046 ⁵
		1.75 \pm 0.93 ⁴				
Non-mucinous	60	9.02 \pm 6.14	0.781 ⁴	25	35	
		2.07 \pm 1.37 ⁴				
TNM stage						
I / II	30	5.17 \pm 2.96	< 0.001 ²	2	28	< 0.001 ⁵
		1.57 \pm 0.80 ⁴				
III / IV	43	10.57 \pm 6.27	0.207 ⁴	24	19	
		2.32 \pm 1.50 ⁴				

¹The value was compared by Student's *t* test; ²The value was compared by Wilcoxon's rank-sum test; ³The relation with variables was evaluated by Pearson χ^2 test; ⁴The value of THKmax according to the cell-types and TNM staging; ⁵The relation with variables was evaluated by continuity correction χ^2 test. SUVmax: Maximal standard uptake value; THKmax: The maximal thickness.

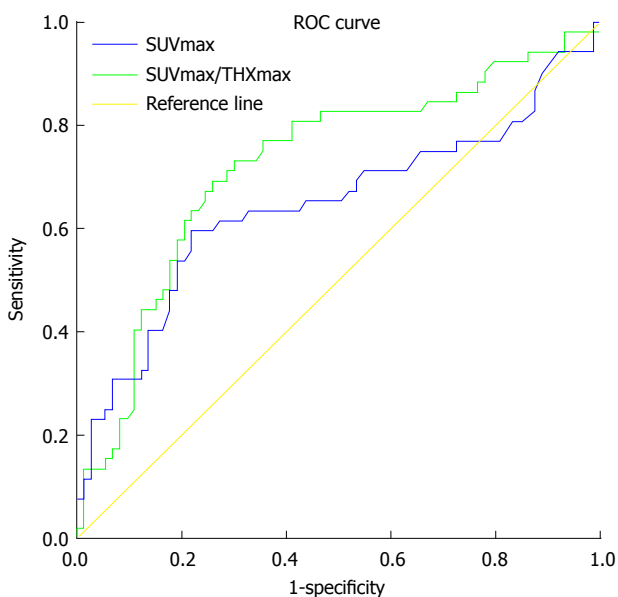


Figure 4 Comparative receiver-operating characteristic curves of SUVmax and SUVmax/THKmax for differential diagnosis between gastric lymphoma and gastric carcinoma. ROC: Receiver-operating characteristic; SUVmax: Maximal standard uptake value; THKmax: Maximal thickness.

should for SUVmax or SUVmax/THKmax for differential diagnosis between gastric lymphoma and gastric carcinoma (Figure 4). The AUC was 0.645 (95%CI: 0.540-0.750) and 0.725 (95%CI: 0.631-0.819). This suggests a more acceptable discrimination of SUVmax/THKmax in comparison with that of SUVmax alone, with a best cutoff threshold at 10.4 and 5.9 for SUVmax and SUVmax/THKmax, respectively. There was no profound difference in specificity (0.781 vs 0.740) for SUVmax and SUVmax/THKmax. The corresponding sensitivity significantly increased from 0.596 to 0.692 when SUVmax alone was replaced by

SUVmax/THKmax to evaluate the diagnostic performance of ¹⁸F-FDG PET/CT in the differential diagnosis of gastric lymphoma and gastric carcinoma.

DISCUSSION

Gastric carcinoma and gastric lymphoma are the two most commonly encountered malignancies in the stomach. ¹⁸F-FDG PET/CT examination is a well-recognized noninvasive imaging modality in staging and treatment response evaluation for gastric wall lesions, including in gastric lymphoma and gastric carcinoma^[12,20]. The advantages of ¹⁸F-FDG PET/CT over conventional imaging techniques have been well characterized in numerous studies^[21,22]. However, few studies have investigated the application of ¹⁸F-FDG PET/CT in the initial differential diagnosis of gastric lymphoma and gastric carcinoma. We aimed to distinguish gastric lymphoma from gastric carcinoma through assessing ¹⁸F-FDG uptake pattern and intensity in gastric wall lesions.

Regarding the ¹⁸F-FDG PET/CT pattern, gastric lymphoma patients predominantly presented with diffuse/segmental tracer uptake (type I and type II), whereas gastric carcinoma patients showed mainly local tracer uptake (type III). ¹⁸F-FDG uptake intensity, measured by SUVmax or SUVmax/THKmax, was significantly higher in patients with gastric lymphoma than gastric carcinoma. Consequently, higher SUVmax and larger SUVmax/THKmax suggest that gastric lymphoma is more likely. Furthermore, the presence of splenomegaly or involvement of lymph nodes in the retroperitoneal space below the renal hilus may provide additional clues in diagnosing gastric lymphoma. More importantly, SUVmax/THKmax was a more reliable indicator to distinguish gastric lymphoma

from gastric carcinoma compared to SUVmax alone.

As expected, DLBCL and MALT lymphomas accounted for the majority of gastric lymphoma subtypes, and the gastric carcinoma patients were divided into mucinous adenocarcinoma and non-mucinous adenocarcinoma subgroups to facilitate comparison with gastric lymphoma patients. MALT lymphoma is indolent, and usually develops as local lesions. Gastric MALT lymphoma detected by ^{18}F -FDG PET/CT has been widely studied, as the stomach is the most commonly involved organ^[11,16,17]. However, there are controversial results with regard to the usefulness of PET/CT scan in the diagnosis of gastric MALT lymphoma. Some studies have suggested that ^{18}F -FDG PET/CT is not a useful imaging method in patients with low-grade lymphoma, such as MALT lymphoma, as ^{18}F -FDG uptake in MALT lymphoma is lower than in aggressive lymphoma, such as DLBCL^[12,15]. Especially for gastric MALT lymphoma, the sensitivity of ^{18}F -FDG PET/CT was low because of the physiological or inflammatory FDG accumulation in the stomach, which usually resulted in false-negative diagnosis. Subsequently, ^{18}F -FDG PET/CT was shown to be useful for evaluating the protrusion type of gastric MALT lymphoma in which mass lesions are formed^[16]. In addition, diffuse or local uptake can occur in chronic-gastritis-like type or depressed type of gastric MALT lymphoma, so endoscopic biopsy is recommended even if the gastroscopy findings suggest chronic gastritis^[16-19,23]. Explanations for this discrepancy have been proposed, including the presence of heterogeneous cell populations in gastric MALT lymphoma^[11]; the partial volume effect of mucosal or small lesions of gastric MALT lymphoma detected by endoscopy^[16,17]; and gastric MALT lymphoma existing in combination with DLBCL, or transformation into DLBCL during follow-up^[25,26]. So, MALT lymphoma with emerging foci of intense ^{18}F -FDG uptake are susceptible to conversion to DLBCL.

MALT lymphoma is generally considered to be a non- ^{18}F -FDG-avid type of lymphoma due to its small volume and indolent behavior^[27]. Plasmacytic differentiation (PCD) has been recently suggested as an important factor influencing the detection rate of MALT lymphoma by ^{18}F -FDG PET/CT^[28]. In contrast, Tsukamoto *et al.*^[29] demonstrated no significant effect of PCD in MALT lymphoma detection. The differences among the studies could be explained by the different stages of the recruited cases, even among those with the same pathological subtype. Unfortunately, we could not confirm the critical role of PCD in ^{18}F -FDG PET/CT evaluation of gastric MALT lymphoma due to the small population size and predominance of PCD in extragastric MALT lymphoma^[29].

To facilitate comparison with gastric lymphoma, we did not further classify subgroups of gastric carcinoma (mucinous vs non-mucinous). As indicated by several previous studies, gastric mucinous and signet ring cell adenocarcinoma frequently present with significantly low ^{18}F -FDG intensity due to low expression of glucose transporter-1 and low glucose metabolism. On the

contrary, the non-mucinous adenocarcinoma subgroup of gastric carcinoma patients exhibited markedly higher FDG uptake compared with the mucinous adenocarcinoma subgroup because of higher metabolic activity^[30].

SUV is a semi-quantitative measure of the normalized concentration of radioactivity in a lesion, and SUVmax is one of the most widely used parameters as an indicator of lesions with a high metabolic rate^[31]. However, SUVmax is a single voxel value that shows the highest intensity of ^{18}F -FDG uptake within the region of interest and may not represent total tumor metabolism^[32]. Instead of SUVmax, volumetric parameters of metabolism such as metabolic tumor volume (MTV) and total lesion glycolysis (TLG) derived from ^{18}F -FDG PET have been recently used for differential analysis, stage stratification survival analysis, and oncogenomic alteration for a variety of malignancies, such as pancreatic cancer^[33], non-small-cell lung cancer^[34] and head and neck cancer^[35]. In view of the potential clinical value of MTV and TLG in the diagnostic performance of PET/CT, we will focus on differentiating gastric lymphoma from gastric carcinoma based on these volumetric parameters of metabolism, and perform a correlation analysis between prognosis and MTV or TLG.

On the basis of studies from Wu *et al.*^[18] and Fu *et al.*^[19], we further characterized the differences in ^{18}F -FDG PET/CT findings between gastric lymphoma and gastric carcinoma and added significant information to the previous studies. First, a larger sample size in our study was an advantage. Second, in the analysis of associated clinicopathological features with SUVmax, we performed subgroup analysis based on sex, age, cell type and staging in gastric lymphoma and gastric carcinoma. In contrast, Wu *et al.*^[18] simply analyzed the difference in THKmax and SUVmax of the gastric wall lesions between patients with gastric lymphoma and gastric cancer, with and without extragastric involvement. Third, unlike Fu *et al.*^[19], who focused on the FDG intensity (SUVmax) of primary lesions and abnormalities detected by CT, including THKmax and mucosal ulcerations, our study was more comprehensive. We reviewed and analyzed the PET/CT features of gastric wall lesions including CT-detected abnormalities (THKmax and ulcerations), FDG avidity and involved region, pattern (focal/diffuse), and intensity (SUVmax). In addition, the correlation of SUVmax with gastric clinicopathological variables was investigated by χ^2 test in our study. Finally, a ROC curve analysis was performed to determine the differential diagnostic value of SUVmax/THKmax in gastric lymphoma and gastric carcinoma.

Our study had some limitations. First, the small number of patients, in the gastric lymphoma group. Furthermore, we excluded one case of Burkitt's lymphoma and one case of NK/T cell lymphoma to facilitate obtaining categorical data for the gastric lymphoma patients with different histological subtypes. Second,

the retrospective nature of the present study could not completely rule out bias in the patient selection. In addition, the differences between our and a previous study^[18] are explained by referring to the comparison of maximal thickness between gastric lymphoma and gastric carcinoma. Therefore, the results and conclusions of our study need to be verified by more prospective studies with a large population.

In conclusion, there were differences in ¹⁸F-FDG PET/CT features of gastric lymphoma compared with gastric carcinoma. The former predominantly presented with diffuse/segmental tracer uptake (type I and type II), whereas the latter showed mainly local tracer uptake (type III). Regarding ¹⁸F-FDG uptake intensity, measured by SUVmax or SUVmax/THKmax, a higher SUVmax and a larger SUVmax/THKmax suggest that gastric lymphoma is more likely. In addition, SUVmax/THKmax was a more reliable indicator for differentiation of gastric lymphoma from gastric carcinoma in comparison with SUVmax alone.

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COMMENTS

Background

The usefulness of ¹⁸F-fluorodeoxyglucose positron emission tomography/computed tomography (¹⁸F-FDG PET/CT) in a variety of malignancies is well established. However, the role of ¹⁸F-FDG PET/CT in gastric lymphomas or gastric carcinomas is challenging due to the physiologic FDG uptake in the stomach and difference in the level of tracer activity in various pathological subtypes.

Research frontiers

To the best of our knowledge, there have been few studies focusing on the imaging differences between gastric lymphomas and gastric carcinomas using ¹⁸F-FDG PET/CT, and divergence also existed in some previous studies. The purpose of the present investigation was to distinguish gastric lymphomas from gastric carcinomas based on the characteristics of PET/CT evaluation for gastric wall lesions.

Innovations and breakthroughs

Gastric lymphomas predominantly presented with diffuse/segmental ¹⁸F-FDG uptake, whereas gastric carcinomas mainly with local uptake, and a higher SUVmax and a larger SUVmax/THKmax. Compared to SUVmax alone, SUVmax/THKmax showed a better performance in the differential diagnosis between gastric lymphomas and gastric carcinomas.

Applications

This study indicated significant differences in the ¹⁸F-FDG PET/CT features between gastric lymphomas and gastric carcinomas. The findings can help differentiate gastric lymphomas from gastric carcinomas based on the PET/CT evaluation.

Terminology

¹⁸F-FDG PET/CT imaging and evaluation with SUVmax/THKmax parameters in gastric wall lesions were considered to be valuable in the differentiation of gastric lymphomas from gastric carcinomas.

Peer-review

This is an interesting study that compared differences in the PET/CT findings between the gastric lymphoma and gastric carcinoma.

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Observational Study

Risk factors for postoperative pancreatic fistula: Analysis of 539 successive cases of pancreaticoduodenectomy

Bing-Yang Hu, Tao Wan, Wen-Zhi Zhang, Jia-Hong Dong

Bing-Yang Hu, Tao Wan, Wen-Zhi Zhang, Jia-Hong Dong, Institute and Hospital of Hepatobiliary Surgery, Chinese PLA General Hospital, Beijing 100853, China

Jia-Hong Dong, Hepato-Pancreato-Biliary Center, Beijing Tsinghua Changgung Hospital, Beijing 102218, China

Author contributions: Hu BY and Dong JH contributed equally to this work; Hu BY and Dong JH designed the research; Hu BY collected and analyzed the data and drafted the manuscript; Zhang WZ and Wan T contributed analytical tools; all authors have read and approved the final version to be published.

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Correspondence to: Dr. Jia-Hong Dong, Institute and Hospital of Hepatobiliary Surgery, Chinese PLA General Hospital; Hepato-Pancreato-Biliary Center, Beijing Tsinghua Changgung Hospital, 168 Litang Road, Beijing 102218, China. zhangwenzhi301301@163.com
Telephone: +86-10-66938331
Fax: +86-10-68241383

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Abstract

AIM

To analyze the risk factors for pancreatic fistula after pancreaticoduodenectomy.

METHODS

We conducted a retrospective analysis of 539 successive cases of pancreaticoduodenectomy performed at our hospital from March 2012 to October 2015. Pancreatic fistula was diagnosed in strict accordance with the definition of pancreatic fistula from the International Study Group on Pancreatic Fistula. The risk factors for pancreatic fistula were analyzed by univariate analysis and multivariate logistic regression analysis.

RESULTS

A total of 269 (49.9%) cases of pancreatic fistula occurred after pancreaticoduodenectomy, including 71 (13.17%) cases of grade A pancreatic fistula, 178 (33.02%) cases of grade B, and 20 (3.71%) cases of grade C. Univariate analysis showed no significant correlation between postoperative pancreatic fistula (POPF) and the following factors: age, hypertension, alcohol consumption, smoking, history of upper abdominal surgery, preoperative jaundice management, preoperative bilirubin, preoperative albumin, pancreatic duct drainage, intraoperative blood loss, operative time, intraoperative blood transfusion, Braun anastomosis, and pancreaticoduodenectomy (with or without pylorus preservation). Conversely, a significant correlation was

observed between POPF and the following factors: gender (male *vs* female: 54.23% *vs* 42.35%, $P = 0.008$), diabetes (non-diabetic *vs* diabetic: 51.61% *vs* 39.19%, $P = 0.047$), body mass index (BMI) (≤ 25 *vs* > 25 : 46.94% *vs* 57.82%, $P = 0.024$), blood glucose level (≤ 6.0 mmol/L *vs* > 6.0 mmol/L: 54.75% *vs* 41.14%, $P = 0.002$), pancreaticojejunal anastomosis technique (pancreatic duct-jejunum double-layer mucosa-to-mucosa pancreaticojejunal anastomosis *vs* pancreatic-jejunum single-layer mucosa-to-mucosa anastomosis: 57.54% *vs* 35.46%, $P = 0.000$), diameter of the pancreatic duct (≤ 3 mm *vs* > 3 mm: 57.81% *vs* 38.36%, $P = 0.000$), and pancreatic texture (soft *vs* hard: 56.72% *vs* 29.93%, $P = 0.000$). Multivariate logistic regression analysis showed that gender (male), BMI > 25 , pancreatic duct-jejunum double-layer mucosa-to-mucosa pancreaticojejunal anastomosis, pancreatic duct diameter ≤ 3 mm, and soft pancreas were risk factors for pancreatic fistula after pancreaticoduodenectomy.

CONCLUSION

Gender (male), BMI > 25 , pancreatic duct-jejunum double-layer mucosa-to-mucosa pancreaticojejunal anastomosis, pancreatic duct diameter ≤ 3 mm, and soft pancreas were risk factors for pancreatic fistula after pancreaticoduodenectomy.

Key words: Pancreaticoduodenectomy; Pancreatic fistula; Pancreaticojejunal anastomosis; Pancreatic duct; Complications

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Core tip: Pancreaticoduodenectomy remains the standard surgical approach for tumors involving the lower bile duct, the pancreatic head, the duodenal papilla, and the ampulla. This operation is considered risky because of high rates of postoperative mortality and complications. In this study, we collected a large sample of 539 cases and analyzed several potential risk factors for pancreatic fistula. A statistical analysis of the case data showed that gender (male), pancreatic duct-jejunum double-layer mucosa-to-mucosa pancreaticojejunal anastomosis, pancreatic duct diameter ≤ 3 mm, and soft pancreas were risk factors for pancreatic fistula after pancreaticoduodenectomy.

Hu BY, Wan T, Zhang WZ, Dong JH. Risk factors for postoperative pancreatic fistula: Analysis of 539 successive cases of pancreaticoduodenectomy. *World J Gastroenterol* 2016; 22(34): 7797-7805 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i34/7797.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i34.7797>

INTRODUCTION

Pancreaticoduodenectomy is the primary treatment for malignant tumors involving the pancreatic head, the lower bile duct, and the duodenal ampulla^[1,2]. The safety of pancreaticoduodenectomy has been greatly improved; however, perioperative mortality rate still ranges from 0%-5%^[3-5]. Studies have reported that the incidence of pancreatic fistula after pancreaticoduodenectomy is approximately 11.4%-64.3%^[6-16]. Pancreatic fistula remains the most common complication after pancreaticoduodenectomy, and this complication prolongs hospital stays and leads to high medical expenses. Pancreatic fistula is associated with delayed gastric emptying, abdominal abscesses, infection at the incision, sepsis, and bleeding after pancreaticoduodenectomy^[17-20]. Several approaches may reduce the incidence of pancreatic fistula after pancreaticoduodenectomy; however, to date, a definitive approach that prevents pancreatic fistula is still lacking^[21-23]. In this study, we conducted a retrospective analysis of 539 successive cases of pancreaticoduodenectomy performed over more than 3 years in the Department of Hepatobiliary Surgery of the PLA General Hospital, China, to determine the potential risk factors for pancreatic fistula.

MATERIALS AND METHODS

Patients and data collection

We reviewed the data from 539 successive cases of pancreaticoduodenectomy performed in the Department of Hepatobiliary Surgery of the PLA General Hospital, China, from March 2012 to October 2015. The following patient data were collected: gender, age, hypertension, diabetes, alcohol consumption, smoking, body mass index (BMI), history of upper abdominal surgery, preoperative jaundice management, blood sugar, preoperative bilirubin, preoperative albumin, pancreatic duct drainage, intraoperative blood loss, intraoperative blood transfusion, operative time, pancreaticojejunal anastomosis technique, Braun anastomosis, pylorus preservation, diameter of the pancreatic duct, and pancreatic texture. Additionally, all postoperative complications and postoperative pathological findings (especially information about pancreatic fistula) were recorded. All 539 cases were included in this comprehensive study.

Preoperative preparation

Prior to surgery, the patients underwent routine tests, examinations, and evaluations of organ function. To date, no uniform standard exists for preoperative jaundice management. We believe that patients with obstructive jaundice who have a poor mental state,

Table 1 Criteria utilized to grade postoperative pancreatic fistula

Grade	A	B	C
Clinical conditions	Well	Often well	Ill appearing/ bad
Specific treatment ¹	No	Yes/no	Yes
US/CT (if obtained)	Negative	Negative/ positive	Positive
Persistent drainage (after 3 wk) ²	No	Usually yes	Yes
Reoperation	No	No	Yes
Death related to POPF	No	No	Possibly yes
Signs of infection	No	Yes	Yes
Sepsis	No	No	Yes
Readmission	No	Yes/no	Yes/no

¹Partial (peripheral) or total parenteral nutrition, antibiotics, enteral nutrition, somatostatin analogue, and/or minimally invasive drainage;

²With or without a drain *in situ*. CT: Computed tomography; POPF: Postoperative pancreatic fistula; US: Ultrasonography.

Table 2 Disease composition

Pathological type	n
Pancreatic head cancer	126
Neuroendocrine tumor of the pancreatic head	12
Benign tumor of the pancreatic head	12
Solid pseudopapillary tumor of the pancreatic head	16
Autoimmune pancreatitis	3
Chronic pancreatitis	11
Lower bile duct cancer	145
Benign tumor of the lower bile duct	13
Ampullary cancer	76
Benign ampullary tumor	6
Duodenal stromal tumor	7
Duodenal cancer	10
Chronic mucosal inflammation of the descending duodenum	3
Duodenal papillary cancer	90
Benign duodenal papillary tumor	6
Duodenal papillary neuroendocrine tumor	3
Total	539

severe dehydration, poor nutrition, or severe jaundice should undergo jaundice management and supportive therapy to improve their nutritional intake and replenish fluids. Patients should subsequently undergo surgery after their general condition has improved.

Surgical approach

In this study, 275 patients underwent classic pancreaticoduodenectomy, of whom 13 had portal vein resection and reconstruction. Additionally, 264 patients underwent pylorus-preserving pancreaticoduodenectomy, of whom six had portal vein resection and reconstruction. Child's technique (pancreaticojejunal anastomosis, biliary-jejunal anastomosis, and gastro-jejunal anastomosis in sequential order) was used for the gastrointestinal reconstruction. A support tube was placed in the pancreatic duct of all patients. The support tube was drained *via* the jejunal loop to outside the body in 127 patients. In the remaining patients,

the tube was placed in the jejunum *via* biliary-jejunal anastomosis. The following two approaches were used for pancreaticojejunal anastomosis: (1) pancreatic duct-jejunum double-layer mucosa-to-mucosa pancreaticojejunal anastomosis in 398 patients; and (2) pancreatic-jejunum single-layer mucosa-to-mucosa anastomosis in 141 patients.

Classification and detailed definition of postoperative pancreatic fistula

Pancreatic fistula was defined according to the International Study Group on Pancreatic Fistula (ISGPF) as any measurable volume of drainage fluid output *via* operatively or postoperatively placed drains on or after postoperative day 3 with amylase content greater than three times the upper normal serum value. Three grades of pancreatic fistulas were determined according to their clinical severity. The grades were determined only after complete healing of the fistula (Table 1)^[24].

Statistical analysis

All clinical data were entered into an Excel spreadsheet, and SPSS 19.0 software was used for statistical analyses. Measurement data are expressed as the mean \pm SD. A *t*-test was performed for between-group comparisons. Categorical variables were analyzed using Fisher's exact test and the χ^2 test. All variables were incorporated into a univariate analysis. $P < 0.05$ was considered statistically significant. Statistically significant variables demonstrated in the univariate analysis were incorporated into a multivariate logistic regression analysis to identify the independent risk factors for pancreatic fistula after pancreaticoduodenectomy.

RESULTS

Overall characteristics of patients and complications

This study included 343 male patients and 196 female patients with a mean age of 56.42 ± 10.75 years. The mean hospital stay was 30.03 ± 8.86 d. The condition (confirmed by postoperative pathology) of the 539 patients is shown in Table 2. Among the 539 patients, 349 (64.75%) experienced complications, and 269 (49.9%) had POPF, including 71 (13.17%) cases of grade A pancreatic fistula, 178 (33.02%) cases of grade B, and 20 (3.71%) cases of grade C. Additionally, 198 (36.73%) patients had clinically relevant POPFs. The following complications were identified: 25 (4.64%) cases of abdominal bleeding, 59 (10.95%) cases of bile leakage, 120 (22.63%) cases of delayed gastric emptying, 9 (1.67%) cases of pancreaticojejunal anastomotic bleeding, 66 (12.24%) cases of abdominal infection, and 45 (8.35%) cases of incision infection. Moreover, 15 (2.78%) patients underwent a second operation, and 6 (1.11%) patients died after surgery due to abdominal bleeding associated with a pancreatic

Table 3 Risk factors for pancreatic fistula according to univariate analysis

Variable	POPF occurrence		χ^2	P value
	Yes	No		
Sex			7.042	0.008
Male	186	157		
Female	83	113		
Age (yr)			2.132	0.144
≥ 60	99	116		
< 60	170	154		
Body mass index (kg/m ²)			5.066	0.024
> 25	85	62		
≤ 25	184	208		
Hypertension			0.938	0.332
Yes	62	53		
No	207	217		
Diabetes mellitus			3.941	0.047
Yes	29	45		
No	240	225		
Drinking history			0.169	0.681
Yes	77	73		
No	193	197		
Smoking history			1.426	0.232
Yes	78	66		
No	191	204		
Epigastrium surgery			0.640	0.424
Yes	18	23		
No	251	247		
Preoperative biliary drainage			0.406	0.524
Yes	65	59		
No	204	211		
Preoperative total bilirubin ($\mu\text{mol/L}$)			0.378	0.539
> 171	73	67		
≤ 171	196	203		
Serum albumin (g/L)			0.000	0.985
< 35	41	41		
≥ 35	228	229		
Blood glucose (mmol/L)			9.157	0.002
≤ 6.0	190	157		
> 6.0	79	113		
Pancreaticojejunostomy			20.323	0.000
Double-layer mucosa-to-mucosa	229	169		
Single-layer mucosa-to-mucosa	50	91		
Blood loss (mL)			0.134	0.715
> 600	34	37		
≤ 600	235	225		
Pancreatic duct diameter (mm)			19.687	0.000
≤ 3	185	135		
> 3	84	135		
Pylorus-preserving			0.017	0.897
Yes	131	133		
No	138	137		
Pancreatic duct drainage			0.016	0.900
External	64	63		
Enteral	205	207		
Intraoperative blood infusion			1.310	0.252
Yes	36	46		
No	233	224		
Operative time (min)			0.299	0.584
> 300	196	191		
≤ 300	73	79		
Braun anastomosis			2.274	0.132
Yes	78	94		
No	192	175		
Pancreatic texture			29.330	0.000
Soft	228	174		
Hard	41	96		

fistula.

Univariate analysis

Univariate analysis showed no significant correlation between POPF and the following factors: age, hypertension, alcohol consumption, smoking, history of upper abdominal surgery, preoperative jaundice management, preoperative bilirubin, preoperative albumin, pancreatic duct drainage, intraoperative blood loss, operative time, intraoperative blood transfusion, Braun anastomosis, and pancreaticoduodenectomy (with or without pylorus preservation). Conversely, a significant correlation was observed between POPF and the following factors: gender (male vs female: 54.23% vs 42.35%, $P = 0.008$), diabetes (non-diabetic vs diabetic: 51.61% vs 39.19%, $P = 0.047$), BMI (≤ 25 vs > 25 : 46.94% vs 57.82%, $P = 0.024$), blood glucose level (≤ 6.0 mmol/L vs > 6.0 mmol/L: 54.75% vs 41.14%, $P = 0.002$), pancreaticojejunal anastomosis technique (pancreatic duct-jejunum double-layer mucosa-to-mucosa pancreaticojejunal anastomosis vs pancreatic-jejunum single-layer mucosa-to-mucosa anastomosis: 57.54% vs 35.46%, $P = 0.000$), diameter of the pancreatic duct (≤ 3 mm vs > 3 mm: 57.81% vs 38.36%, $P = 0.000$), and pancreatic texture (soft vs hard: 56.72% vs 29.93%, $P = 0.000$) (Table 3).

Multivariate logistic regression analysis

The risk factors for pancreatic fistula (gender, diabetes, BMI, blood glucose level, pancreaticojejunal anastomosis technique, the diameter of the pancreatic duct, and pancreatic texture) demonstrated in the univariate analysis were incorporated into the logistic regression analysis. The results showed that gender (male), BMI > 25 , pancreatic duct-jejunum double-layer mucosa-to-mucosa pancreaticojejunal anastomosis, pancreatic duct diameter ≤ 3 mm, and soft pancreas were risk factors for pancreatic fistula after pancreaticoduodenectomy (Table 4).

DISCUSSION

The causes of pancreatic fistula include pancreaticojejunal anastomotic leak, leak from pancreatic resection, leak associated with damage to the pancreatic capsule, and leak *via* the puncture channel. Pancreatic fistula after pancreaticoduodenectomy is a common and serious complication and the most important cause of subsequent complications and death after this procedure^[8,24-26]. The dilemma of pancreatic fistula after pancreaticoduodenectomy has not yet been resolved^[27]. Currently, researchers believe that the following factors are related to pancreatic fistula: gender, age, preoperative jaundice, intraoperative blood loss, operative time, pancreatic texture, BMI, diameter of the main pancreatic duct, and pancreaticojejunal anastomosis^[14,28-32]. Peng et

Table 4 Logistic regression for the predictors of pancreatic fistula following pancreaticoduodenectomy

Variable	B	SE	Wals	P value	OR	95%CI
Sex	0.579	0.196	8.688	0.003	1.784	1.214-2.622
Body mass index	0.518	0.213	5.941	0.015	1.679	1.107-2.546
Pancreaticojejunostomy	0.743	0.217	11.723	0.001	2.102	1.374-3.216
Pancreatic duct diameter	0.724	0.192	14.254	0.000	2.062	1.416-3.003
Pancreatic texture	1.115	0.227	24.102	0.000	3.048	1.953-4.757

al^[33] suggested that bundled pancreaticogastrostomy was a safe and effective anastomosis technique to prevent the leakage of pancreatic juice from pancreaticojejunal anastomosis. Shubert *et al*^[34] believed that the clinical risk score for pancreatic fistula (CRS-PF) could effectively predict pancreatic fistula after pancreaticoduodenectomy. In this study, multivariate logistic regression analysis showed that gender (male), BMI > 25, pancreatic duct-jejunum double-layer mucosa-to-mucosa pancreaticojejunal anastomosis, pancreatic duct diameter ≤ 3 mm, and soft pancreas were risk factors for pancreatic fistula after pancreaticoduodenectomy.

Kawai retrospectively analyzed the perioperative data from 1239 patients treated at 11 medical facilities from 2005 to 2009 and summarized that the male gender was a risk factor for pancreatic fistula after pancreaticoduodenectomy^[35]. This study included 343 male patients (POPF rate: 54.23%) and 196 female patients (POPF rate: 42.35%). Univariate analysis showed that the difference in the POPF rate was significant ($P = 0.008$), suggesting that male patients were at a higher risk than female patients for the development of pancreatic fistula after pancreaticoduodenectomy. Additionally, multivariate logistic regression analysis showed that the difference was significant ($P = 0.003$), suggesting that gender (male) was a risk factor for pancreatic fistula after pancreaticoduodenectomy. The odds ratio (OR = 1.784; 95%CI: 1.214-2.622) showed that the risk of developing pancreatic fistula after pancreaticoduodenectomy was 1.784-fold higher in male patients than in female patients.

El Nakeeb *et al*^[36] analyzed 471 cases of pancreaticoduodenectomy and found that BMI > 25 was a risk factor for POPF. Gaujoux *et al*^[28] analyzed 100 successive cases of pancreaticoduodenectomy and similarly found that BMI > 25 was a risk factor for pancreatic fistula after pancreaticoduodenectomy. In our study, 392 patients had a BMI ≤ 25 (POPF rate: 46.94%), and 147 patients had a BMI > 25 (POPF rate: 57.82%). Univariate analysis showed that the difference in the POPF rates was significant ($P = 0.024$), suggesting that patients with a BMI > 25 were at a higher risk of developing pancreatic fistula after pancreaticoduodenectomy than patients with a BMI ≤ 25. Additionally, multivariate logistic regression analysis showed that the difference was significant ($P = 0.015 < 0.05$), which indicated that

BMI > 25 was a risk factor for pancreatic fistula after pancreaticoduodenectomy. The OR (1.679, 95%CI: 1.107-2.546) showed that the risk of developing a pancreatic fistula after pancreaticoduodenectomy was 1.679-fold higher in patients with a BMI > 25 than in patients with a BMI ≤ 25. The higher incidence of pancreatic fistula after pancreaticoduodenectomy in patients with a BMI > 25 may be associated with the following factors: increased difficulty in exposing the pancreas during surgery due to a higher volume of abdominal fat and peripancreatic fat, a higher risk of damage to the pancreatic capsule during separation due to a soft and brittle pancreas, and a higher risk of pancreatic leakage caused by damage to the pancreatic tissue and fine pancreatic ducts due to suturing and knotting during pancreaticojejunal anastomosis.

Pancreaticojejunal anastomosis is a critical step during pancreaticoduodenectomy and affects the surgical outcome. However, pancreaticojejunal anastomosis is a complex procedure during pancreaticoduodenectomy, and the choice of an appropriate pancreaticojejunal anastomosis technique should reduce the incidence of pancreatic fistula^[33,37-40]. Fu *et al*^[32] retrospectively analyzed 532 cases of pancreaticoduodenectomy and found that the pancreaticojejunal anastomosis technique was a risk factor for pancreatic fistula after pancreaticoduodenectomy. In this study, pancreatic duct-jejunum double-layer mucosa-to-mucosa pancreaticojejunal anastomosis was performed in 398 patients (POPF rate: 57.54%), and pancreatic-jejunum single-layer mucosa-to-mucosa anastomosis was performed in 141 patients (POPF rate: 35.46%). Univariate analysis showed that the difference in the POPF rates was significant ($P = 0.000$), suggesting that patients who underwent pancreatic duct-jejunum double-layer mucosa-to-mucosa pancreaticojejunal anastomosis were at a higher risk of developing pancreatic fistula after pancreaticoduodenectomy than patients who underwent pancreatic-jejunum single-layer mucosa-to-mucosa anastomosis. Additionally, multivariate logistic regression analysis showed that the difference was significant ($P = 0.001$), suggesting that pancreatic duct-jejunum double-layer mucosa-to-mucosa pancreaticojejunal anastomosis was an independent risk factor for pancreatic fistula after pancreaticoduodenectomy. The OR (2.102, 95%CI: 1.374-3.216) indicated that the risk of developing a

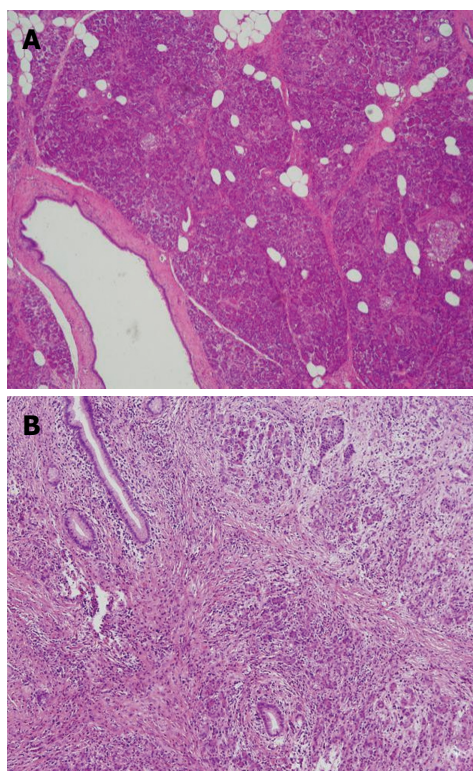


Figure 1 Photomicrographs of the pathological examination for pancreatic fibrosis. A: No significant fibrosis; B: Severe fibrosis (hematoxylin-eosin staining; original magnification, $\times 100$).

pancreatic fistula after pancreaticoduodenectomy was 2.102-fold higher in patients who underwent pancreatic duct-jejunum double-layer mucosa-to-mucosa pancreaticojejunal anastomosis than in patients who underwent pancreatic-jejunum single-layer mucosa-to-mucosa anastomosis. The higher incidence of pancreatic fistula after pancreaticoduodenectomy in patients who underwent pancreatic duct-jejunum double-layer mucosa-to-mucosa pancreaticojejunal anastomosis may be related to the following factors: use of the 6-0 PDS II suture during pancreatic duct-jejunum anastomosis because the fine suture can easily cut the pancreatic duct during suturing and knotting, thereby resulting in a pancreaticojejunal anastomotic leak, and the use of the 5-0 PDS II suture to suture the pancreatic section and the jejunal seromuscular layer because this suture can easily cut the pancreas and fine pancreatic ducts during suturing and may not tightly secure the pancreatic section and the jejunal seromuscular layer, thereby resulting in pancreatic leakage and leakage from the fine pancreatic ducts. In contrast, the 4-0 Vicryl suture is used for full-layer suturing of the pancreas, the pancreatic duct, and the jejunum during pancreatic-jejunum single-layer mucosa-to-mucosa anastomosis; therefore, the suture is secure and reduces the risk of cutting the pancreas. Moreover, the jejunal seromuscular layer covers the entire pancreatic section and presses the fine pancreatic ducts at the pancreatic

section, thereby reducing pancreatic leakage^[38].

A soft pancreas is a risk factor for pancreatic fistula after pancreaticoduodenectomy^[35-37,41,42]. The pancreatic stumps of all cases were submitted for pathological diagnosis of the degree of pancreatic fibrosis. All cases of pancreatic texture were divided into two groups (normal soft pancreas with no significant fibrosis, as shown in Figure 1A, and hard pancreas with fibrosis, as shown in Figure 1B). In this study, 402 patients had a soft pancreas (POPF rate: 56.72%), and 137 patients had a hard pancreas (POPF rate: 29.93%). Univariate analysis showed that the difference in the POPF rates was significant ($P = 0.000$), suggesting that patients with a soft pancreas were at a higher risk of developing a pancreatic fistula after pancreaticoduodenectomy than patients with a hard pancreas. Additionally, multivariate logistic regression analysis demonstrated that the difference was significant ($P = 0.000$), which indicated that a soft pancreas was an independent risk factor for pancreatic fistula after pancreaticoduodenectomy. The OR (3.048, 95%CI: 1.953-4.757) showed that the risk of developing a pancreatic fistula after pancreaticoduodenectomy was 3.048-fold higher in patients with a soft pancreas than in patients with a hard pancreas. The higher incidence of pancreatic fistula after pancreaticoduodenectomy in patients with a soft pancreas may be related to insecure suturing and knotting, which can result in unsatisfactory pancreaticojejunal anastomosis and a higher risk of damage to the pancreatic tissue and fine pancreatic ducts during suturing and knotting of a soft pancreas, resulting in pancreatic leakage. The lower incidence of pancreatic fistula after pancreaticoduodenectomy in patients with a hard pancreas may be related to pancreatic exocrine dysfunction due to prolonged pancreatic duct obstruction and pancreatic fibrosis, secure pancreaticojejunal anastomosis, and obstructed minor ducts at the cut-surface of the hard pancreas, and this could help reduce POPF^[43] and risk of damage to the pancreatic tissue and fine pancreatic ducts during suturing and knotting. Pancreatic texture is the most significant single predictor of POPF, and clinicians should select a pancreaticojejunal anastomosis technique based on the texture of the pancreas to reduce the incidence of POPF^[44].

Pancreatic duct diameter ≤ 3 mm is a risk factor for pancreatic fistula after pancreaticoduodenectomy^[14,37]. In this study, the diameter of the pancreatic duct was ≤ 3 mm in 320 patients (POPF rate: 57.81%) and > 3 mm in 219 patients (POPF rate: 38.36%). Univariate analysis showed that the difference in the POPF rates was significant ($P = 0.000$), suggesting that patients with a pancreatic duct diameter ≤ 3 mm were at a higher risk of developing a pancreatic fistula after pancreaticoduodenectomy than patients with a pancreatic duct diameter > 3 mm. Additionally, multivariate logistic regression analysis indicated that

the difference was significant ($P = 0.000$), suggesting that pancreatic duct diameter ≤ 3 mm was an independent risk factor for pancreatic fistula after pancreaticoduodenectomy. The OR (2.062, 95%CI: 1.416-3.003) showed that the risk of developing a pancreatic fistula after pancreaticoduodenectomy was 2.062-fold higher in patients with a pancreatic duct diameter ≤ 3 mm than in patients with a pancreatic duct diameter > 3 mm. The lower incidence of pancreatic fistula after pancreaticoduodenectomy in patients with a pancreatic duct diameter > 3 mm may be related to pancreatic duct obstruction, pancreatic duct fibrosis, pancreatic fibrosis, ease of suturing, and a lower risk of damage to the pancreatic duct during suturing and knotting. As a result, the incidence of pancreatic fistula after pancreaticoduodenectomy was lower in patients with pancreatic duct dilation than in patients without.

Univariate analysis demonstrated that the incidence of pancreatic fistula after pancreaticoduodenectomy was significantly lower in diabetic patients than in non-diabetic patients (39.19% vs 51.61%, $P = 0.047$) and was significantly lower in patients with a blood glucose level > 6.0 mmol/L than in patients with a blood glucose level ≤ 6.0 mmol/L (41.14% vs 54.75%, $P = 0.002$). However, multivariate logistic regression analysis showed that these differences were not significant ($P = 0.268$ and $P = 0.115$, respectively); therefore, diabetes was not a risk factor for POPF.

In conclusion, gender (male), BMI > 25 , pancreatic duct-jejunum double-layer mucosa-to-mucosa pancreaticojejunal anastomosis, pancreatic duct diameter ≤ 3 mm, and soft pancreas were risk factors for pancreatic fistula after pancreaticoduodenectomy.

COMMENTS

Background

Pancreaticoduodenectomy remains the standard surgical treatment for tumors involving the lower bile duct, the pancreatic head, the duodenal papilla, and the ampulla. Pancreaticoduodenectomy is difficult to perform and is associated with high morbidity and mortality. Most complications after pancreaticoduodenectomy are associated with postoperative pancreatic fistula (POPF); however, no definitive approach can prevent pancreatic fistulas.

Research frontiers

The incidence of tumors involving the lower bile duct, the pancreatic head, the duodenal papilla, and the ampulla increases each year, and more patients are undergoing pancreaticoduodenectomy. Surgical techniques and perioperative management are improving; however, the incidence and postoperative mortality of POPF remain high.

Innovations and breakthroughs

Gender (male), BMI > 25 , pancreatic duct-jejunum double-layer mucosa-to-mucosa pancreaticojejunal anastomosis, pancreatic duct diameter ≤ 3 mm, and soft pancreas are risk factors for pancreatic fistula after pancreaticoduodenectomy. Postoperative complications and deaths are related to pancreatic fistulas. For patients with a small pancreatic duct diameter or a soft pancreas, surgeons should select a pancreaticojejunal anastomosis technique that is associated with a lower incidence of POPF.

Applications

POPF was diagnosed in strict accordance with the definition of pancreatic fistula from the ISGPF. POPFs are more common in patients with relevant risk factors, such as male gender, BMI > 25 , pancreatic duct-jejunum double-layer mucosa-to-mucosa pancreaticojejunal anastomosis, pancreatic duct diameter ≤ 3 mm, and soft pancreas. Care must be taken in patients with any of these risk factors, and an appropriate pancreaticojejunal anastomosis technique should be selected based on the texture of the pancreas and the diameter of the pancreatic duct. Patients must be closely monitored after surgery, and patients with a pancreatic fistula must be treated promptly to reduce the risk of fatal complications.

Terminology

Pancreaticoduodenectomy remains the standard surgical treatment for tumors involving the lower bile duct, the pancreatic head, the duodenal papilla, and the ampulla. POPF is common after pancreaticoduodenectomy and is the leading cause of postoperative complications and death following this procedure.

Peer-review

This retrospective study was well designed, and the statistical analysis was highly accurate. The article has a sufficient number of references. The manuscript language is of high quality, and the conclusions of the study are rational. The findings from this study contribute to our understanding of pancreatic fistula after pancreaticoduodenectomy. Readers with an interest in pancreatic fistulas will find this paper beneficial and informative.

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Prospective Study

Hepatocellular carcinoma screening and surveillance in 2293 chronic hepatitis B patients in an endemic area

Teerapat Ungtrakul, Chulabhorn Mahidol, Pattri Chun-on, Charlie Laohapand, Surachate Siripongsakun, Akeanong Worakitsitisatorn, Sirachat Vidhayakorn, Wariya Boonchuay, Jiraporn Dechma, Gaidganok Sornsamdang, Kamonwan Soonklang, Tassanee Sriprayoon, Tawesak Tanwandee, Chirayu U Auewarakul

Teerapat Ungtrakul, Chulabhorn Mahidol, Pattri Chun-on, Charlie Laohapand, Surachate Siripongsakun, Akeanong Worakitsitisatorn, Sirachat Vidhayakorn, Wariya Boonchuay, Jiraporn Dechma, Gaidganok Sornsamdang, Kamonwan Soonklang, Chirayu U Auewarakul, Chulabhorn Hospital, Laksi, Bangkok 10210, Thailand

Chulabhorn Mahidol, Chulabhorn Research Institute, Laksi, Bangkok 10210, Thailand

Chulabhorn Mahidol, Tassanee Sriprayoon, Tawesak Tanwandee, Chirayu U Auewarakul, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkoknoi, Bangkok 10700, Thailand

Author contributions: Ungtrakul T carried out the literature review, study design, statistical analysis, manuscript drafting and revision; Chun-on P, Siripongsakun S, Worakitsitisatorn A and Vidhayakorn S carried out data collection and imaging analyses; Boonchuay W and Dechma J contributed to data collection and patient coordination; Sornsamdang G was responsible for the analysis of serum alpha-fetoprotein and liver function tests; Soonklang K carried out the statistical analysis; Laohapand C and Sriprayoon T contributed to the literature review and study design; Mahidol C, Tanwandee T and Auewarakul CU contributed to proposal development, data analysis and monitoring, and manuscript revision; all authors read and approved the final manuscript.

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Correspondence to: Teerapat Ungtrakul, MD, MSc, Chulabhorn Hospital, 54 Kamphaeng Phet 6, Laksi, Bangkok 10210, Thailand. ungteerapat@yahoo.com
Telephone: +662-5766791
Fax: +662-5766791

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Abstract

AIM

To determine the role of screening and surveillance of hepatocellular carcinoma (HCC) in treatment-naïve chronic hepatitis B (CHB) patients.

METHODS

We recruited 2293 CHB patients (both males and

females; aged 20-65 years). All patients were screened and underwent surveillance using abdominal ultrasonography (AUS) and serum alpha-fetoprotein (AFP) assay every 6 mo. The diagnosis, staging and treatment of HCC followed the American Association for the Study of Liver Diseases practice guidelines and the Barcelona Clinic Liver Cancer guidelines. The exclusion criteria included: decompensated cirrhosis; a history of any cancer in the last 5 years; previous antiviral treatment for CHB; concurrent infection with hepatitis C virus or human immunodeficiency virus; a Karnofsky Performance Status score < 60%; or any medical condition preventing eligibility to complete the protocol. The prevalence and incidence rates of HCC were determined; survival rates were calculated at 3-year post HCC diagnosis. The sensitivity and specificity were calculated on a per-patient basis.

RESULTS

Among 2293 treatment-naïve CHB patients, seven cases had HCC at initial screening, giving a prevalence rate of 305 per 100000 persons; 3.3% were diagnosed with liver cirrhosis, all of which were Child-Pugh class A. With a median follow-up time of 42 (range, 3-48) mo, 10 additional cases were diagnosed with HCC, resulting in an incidence rate of 143 per 100000 persons per year. This burden was as high as that reported in other studies from East Asian countries. All HCC patients were aged ≥ 40 years. Most were at an early stage (Stage 0, A or B); 14/17 cases were successfully treated with surgical resection or radiofrequency ablation, with a high 3-year survival rate of 90%. Hemangioma was the most common focal liver lesion in CHB patients detected by AUS; the main causes of AFP elevation at the initial screening were cirrhosis, increased alanine aminotransferase level and HCC. AUS detected 16/17 HCC cases whereas AFP levels ≥ 20 $\mu\text{g/L}$ at diagnosis were observed in only 7/17 patients, most with a tumor size > 5 cm. For HCC screening and surveillance, AUS had a sensitivity and specificity of 94% and 82%, respectively, whereas the sensitivity and specificity of AFP at a cut-off value of ≥ 20 $\mu\text{g/L}$ were 41% and 98%, respectively. Combined use of AUS and AFP assay did not improve effectiveness.

CONCLUSION

Implementation of active screening and surveillance using AUS to detect early-stage HCC in naïve CHB patients aged ≥ 40 years in an endemic area is of benefit.

Key words: Liver cancer; Ultrasonography; Alpha-fetoprotein; Early detection; Hepatitis B

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Core tip: This large cohort study of 2293 patients revealed a high prevalence rate (305 per 100000 persons) and a high incidence rate (143 per 100000 persons per year) of hepatocellular carcinoma (HCC)

in treatment-naïve Thai chronic hepatitis B (CHB) patients through a screening and surveillance semi-annual ultrasonography program. Most patients were at an early stage (Stage 0, A or B) and were successfully treated, with a high 3-year survival rate of 90%. A national screening policy should thus be implemented in CHB patients residing in a developing country with a high incidence rate of HCC such as Thailand, to prevent late-stage HCC development.

Ungtrakul T, Mahidol C, Chun-on P, Laohapand C, Siripongsakun S, Worakitsitatorn A, Vidhayakorn S, Boonchuay W, Dechma J, Sornsamdang G, Soonklang K, Sriprayoon T, Tanwandee T, Auewarakul CU. Hepatocellular carcinoma screening and surveillance in 2293 chronic hepatitis B patients in an endemic area. *World J Gastroenterol* 2016; 22(34): 7806-7812 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i34/7806.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i34.7806>

INTRODUCTION

Hepatitis B virus (HBV)-associated hepatocellular carcinoma (HCC) is a major health problem in all regions of Thailand, with approximately 12000 new cases per year; most are diagnosed at an advanced stage. The mortality rate is also high with a median survival time of < 1 year^[1-5]. HCC screening and HCC surveillance are defined as a one-time test and repeated tests over time for detecting HCC, respectively^[6]. Abdominal ultrasonography (AUS) and serum alpha-fetoprotein (AFP) measurement are widely accepted as routine HCC screening and surveillance tests in chronic hepatitis B (CHB) patients^[7]. Several previous studies have shown the benefit of HCC screening and surveillance in the detection of early-stage HCC; however, there are conflicting results regarding the efficacy of screening and surveillance concerning improvement in survival^[8-10].

The validity of AFP or AUS or both for the screening and surveillance of HCC remains variable in large-scale screening programs^[11-13]. The sensitivity and specificity of AFP is 40%-60% and 76%-94%, respectively^[14], as compared with 71%-84% and 93%-98%, respectively, for AUS^[15,16]. Currently, international liver societies including the European Association for the Study of the Liver and the American Association for the Study of Liver Diseases recommend screening and surveillance tests in CHB patients who are at high risk of HCC development^[17,18].

At present, no national policy for screening and early detection of HCC is available in Thailand, which is an endemic area for hepatitis B infection. HCC remains the number one cancer killer in the Thai population, affecting both males and females. Screening and surveillance are not accessible in most regions because of the lack of personnel and technology and its associated high costs. Moreover, the majority of CHB

patients in Thailand are unaware of the possible long-term consequences of their conditions, including the development of HCC.

In the present study, we undertook a screening and surveillance program involving treatment-naïve CHB patients using AUS and serum AFP assay to screen for early-stage HCC. The prevalence and incidence rates of HCC were determined. Patients were followed every 6 mo and survival rates were also calculated at 3-year post HCC diagnosis.

MATERIALS AND METHODS

Study population

We enrolled male and female Thai patients, aged 20–65 years, who were serologically positive for hepatitis B surface antigen (s-Ag). The exclusion criteria included: decompensated cirrhosis (Child-Pugh class C or Model for End-stage Liver Disease score > 15); a history of any cancer in the last 5 years; previous antiviral treatment for CHB; concurrent infection with hepatitis C virus infection or human immunodeficiency virus infection; a Karnofsky Performance Status score < 60%; or any medical condition preventing eligibility to complete the protocol (e.g., poor renal function, a serum creatinine level > 1.5 mg/dL, or creatinine clearance < 50 mL/min). An interview questionnaire was utilized to collect the demographic data, clinical data and social determinants. All participants underwent a complete blood count, liver function tests, serum creatinine measurement, prothrombin time measurement, and human immunodeficiency virus antibody testing. Serological tests included AFP assay, quantitative measurement of hepatitis B s-Ag, electrochemiluminescence assay of hepatitis B e-antigen (COBAS 6000/e601 Roche Diagnostics, Mannheim, Germany), electrochemiluminescence assay of anti-HCV (Model e601: Cobas 600, Roche Diagnostics, United States), and alanine aminotransferase (ALT) assay using a serum chemistry auto analyzer (Model 400: COBAS Integra, Roche Diagnostics, United States) using commercial reagents (Cobas Integra ALT: Roche Diagnostics). Serum HBV DNA levels were tested using frozen samples by means of the COBAS AmpliPrep/CoBAS TaqMan HBV test v2.0 (Roche Diagnostics, United States), certified at a lower detection limit of 20 IU/mL of HBV DNA. This study met the guidelines of the Helsinki Declaration and was approved by the Ethical Committee for Human Research of Chulabhorn Research Institute (Certificate No. 18/2553). Written informed consent was obtained from all patients who participated in the study.

Screening and surveillance protocol

AUS examinations were performed by experienced radiologists at the initial screening and every 6 mo thereafter to evaluate the following factors: liver size; caudate/right lobe ratio; liver parenchyma

and surface; space-occupying lesions; portal vein diameter; spleen size; ascites; porto-systemic shunt; bile duct dilatation; and intraabdominal lymphadenopathy. Measurement of HBV DNA, aspartate aminotransferase, alanine aminotransferase (ALT), and AFP levels were repeated at 6-mo intervals. If the serum AFP was ≥ 20 μ g/L or a focal solid liver nodule was detected on AUS, further diagnostic studies were performed including computerized tomography, magnetic resonance imaging, or biopsy of the liver lesion. Importantly, all patients were reminded by our study team to schedule follow-up examinations. Antiviral agents were given if HBV DNA levels were ≥ 2000 IU/mL, with any of the following: (1) ALT > 60 IU/mL; (2) a transient elastography (FibroScan: Echosens, Paris, France) Fibroscan score > 7.2 kPa; or (3) significant fibrosis or cirrhosis on liver biopsy.

HCC was the major outcome in our study. The patients were followed until the time of HCC diagnosis and death. Patients who were lost to follow-up were censored at the time of their last visit to the clinic. HCC was diagnosed using the American Association for the Study of Liver Diseases practice guidelines and the Barcelona Clinic Liver Cancer (BCLC) guidelines were used for tumor staging^[18]. All HCC patients were evaluated for hepatic resection, radiofrequency ablation, chemoembolization or systemic treatment. Additionally, cirrhosis was defined according to the following criteria: a METAVIR fibrosis score from liver biopsy equal to 4, or two consecutive AUS examinations indicating liver cirrhosis or ultrasonographic cirrhosis with a FibroScan score ≥ 10.0 kPa^[19,20].

Statistical analysis

Values were expressed as medians (ranges) and frequency unless otherwise indicated. Patient survival was calculated from the time of HCC diagnosis to the time of death or last follow-up. For evaluation of the usefulness of the screening and surveillance test, the sensitivity and specificity were calculated on a per-patient basis. Patients with a mass lesion on AUS or a serum AFP level > 20 μ g/dL without subsequent HCC confirmation on computed tomography or magnetic resonance imaging scans were recorded as false positive tests. All data were processed and analyzed using Stata/SE v.12 software (StataCorp LP, College Station, TX, United States).

RESULTS

CHB patient characteristics

A total of 2293 CHB patients were included in the study, of whom 1078 (47%) were males with a median age of 41.25 years and 54% were aged > 40 years. The demographic and clinical data are summarized in Table 1. HBV e-Ag negative status with an HBV DNA concentration < 2000 IU/mL was most common and noted in 52% of the patients. This

Table 1 Chronic hepatitis B Patient baseline characteristics *n* (%)

Characteristics	Total (<i>n</i> = 2293)
Male sex	1078 (47.0)
Marital status (married)	1306 (57.2)
Alcohol consumption	683 (29.7)
Tobacco habit	290 (12.6)
Overweight: BMI > 25	854 (37.2)
HBV status	
Hepatitis B e-Ag positive	394 (17.0)
Hepatitis B e-Ag negative with HBV DNA < 2000 IU/mL	1184 (52.0)
Hepatitis B e-Ag negative with HBV DNA ≥ 2000 IU/mL	713 (31.0)
ALT > 60 IU/L	262 (11.4)
Cirrhosis	77 (3.3)

BMI: Body mass index; ALT: Alanine aminotransferase.

was followed by HBV e-Ag negative patients with an HBV DNA concentration of ≥ 2000 IU/mL (31%) and HBV e-Ag positive patients (17%). Elevated ALT levels were observed in 11% of the patients. At enrollment, cirrhosis was diagnosed in 77 cases (3.3%), all of which were scored as Child-Pugh class A; only eight cases had ultrasonographic evidence of portal hypertension.

With respect to protocol adherence, 203 patients withdrew from the surveillance program after completing at least one visit (8%) by our clinical cut-off date for analysis (November 1, 2015). The median number of surveillance tests per patient was 8 (range, 1-8) and 85% of patients were followed up as scheduled.

Prevalence and incidence rates and case characteristics of HCC

HCC was detected in seven cases at the first screening, giving prevalence rates of 305 per 100000 persons. With a median follow-up time of 42 (range, 3-48) mo, 10 additional cases were diagnosed with HCC, resulting in incidence rates of 143 per 100000 persons per year. The characteristics of the HCC subjects are summarized in Table 2. The median age at diagnosis was 57 (range, 40-69) years and half of the subjects also had liver cirrhosis.

The diagnosis of HCC was histologically confirmed in 14 patients. Interestingly, one case (patient no. 15) was diagnosed with a combined hepatocholangiocarcinoma subtype. Of the 17 patients who developed HCC, seven tumors were classified as very early stage (BCLC stage 0) and nine were classified as early stage (BCLC stage A), and only one patient had an intermediate stage tumor (BCLC stage B). Altogether, HCC were resected in 12 cases and two cases underwent radiofrequency ablation. Two out of 12 resectable patients received preoperative transarterial chemoembolization (TACE) because they had large tumors. Furthermore, three cases (patient no. 8, 10 and 15) were inoperable because of excessive portal hypertension, and radio-

frequency ablation was not performed because of a difficult tumor location. No patient was sent for liver transplant in this study.

During follow-up, three of the 17 patients died. One patient died from sepsis and two patients died from HCC. All patient tumors were inoperable at the initial presentation. The 1- and 3-year survival rates were 100% and 90%, respectively. The median survival time was not reached.

Sensitivity and specificity of AFP measurement in the detection of HCC

AFP levels ≥ 20 µg/L at diagnosis were observed in seven of 17 HCC patients, most with a tumor size > 5 cm. The reasons for elevation of the AFP level at initial screening (≥ 20 µg/L) are detailed in Table 3. AFP elevation was caused by pregnancy in two women. In 10 patients, the increased AFP level was accompanied by a transient increase in ALT levels. After treatment with antiviral medication, ALT and AFP levels returned to normal. The remaining 10 cirrhotic patients had a single episode of transient AFP elevation of undetermined cause. The per-patient sensitivity and specificity of AFP in the detection of HCC was 41% and 98%, respectively (Table 4).

Sensitivity and specificity of AUS in the detection of HCC

AUS could detect 16 out of 17 HCC cases with a median tumor size of 2.5 (range, 1.2-7.8) cm. In 2293 subjects, the initial AUS revealed a focal solid liver nodule in 96 patients (4%). Hemangioma was the most common focal hepatic lesion (Table 5). The per-patient sensitivity and specificity of AUS in the detection of HCC in CHB patients was 94% and 82%, respectively. Using AUS in combination with AFP did not increase the sensitivity or specificity (Table 4).

DISCUSSION

A requirement for HCC screening is presently not a national policy in Thailand. The majority of CHB patients are not offered antiviral therapy, leading to very high mortality from associated conditions including HCC. According to studies by Vatanasapt *et al.*^[21], hospital-based 5-year survival rates of HCC cases were only 8.5% in males and 8.3% in females. The present study confirmed that the HCC burden was a major health problem in Thailand, with an urgent need to implement an HCC screening and surveillance program. The prevalence and incidence rates of HCC in our study were as high as those reported in previous studies from other East Asian countries^[22].

In our study, we found that screening and surveillance for HCC using AUS and serum AFP testing every 6 mo led to tumor detection at an operable and early stage in 80% of cases; this resulted in patients having a higher chance of receiving curative treatment.

Table 2 Hepatocellular carcinoma patient characteristics

Patient No.	Age (yr)	Sex	Time from entry to diagnosis (mo)	Tumor No.	Size (cm)	Cirrhosis	BCLC stage	AFP at diagnosis (μg/L)	Treatment
1	50	M	At entry	1	6.5	Y	A	391	Segmental resection
2	63	M	At entry	2	6.4	N	B	8419	TACE then segmental resection
3	54	M	At entry	1	4.5	Y	A	4	Hepatectomy
4	57	M	At entry	1	7.8	Y	A	25	TACE, PVE then hepatectomy
5	44	M	At entry	1	1.7	N	0	4	Segmental resection
6	51	M	At entry	1	6.5	N	A	24	Hepatectomy
7	45	F	At entry	1	4.5	N	A	55	Hepatectomy
8	45	F	6	1	1.4	Y	0	5	TACE
9	62	F	6	2	1.3	Y	0	13	RFA
10	61	M	6	1	4.7	Y	A	22	TACE
11	57	F	12	2	4.0	N	A	15	Segmental resection
12	66	M	24	1	1.7	Y	0	3	Segmental resection
13	40	F	24	1	1.4	N	0	< 1	Segmental resection
14	69	F	30	1	1.4	N	0	2	Segmental resection
15	61	F	39	1	2.2	Y	A	3020	SBRT, TACE
16	64	M	39	1	1.7	Y	0	8.2	RFA
17	48	M	45	1	2.6	N	A	2.5	Segmental resection

AFP: Alpha-fetoprotein; BCLC: Barcelona Clinic Liver Cancer; TACE: Transarterial chemoembolization; PVE: Portal vein embolization; RFA: Radiofrequency ablation; SBRT: Stereotactic body radiation therapy.

Table 3 Cause of alpha-fetoprotein elevation at the initial screening and the number of subjects

Cause	No. of subjects
Hepatocellular carcinoma	5
Cirrhosis	10
Increased ALT levels	10
Pregnancy	2

ALT: Alanine aminotransferase.

Table 4 Performance of screening and surveillance tests for hepatocellular carcinoma

Test	Sensitivity (%)	Specificity (%)
AFP	7/17 (41)	2247/2276 (98)
Ultrasound	16/17 (94)	1879/2276 (82)
Ultrasound and AFP	16/17 (94)	1872/2276 (82)

AFP: Alpha-fetoprotein.

Table 5 Diagnosis of focal liver nodules at initial screening using abdominal ultrasonography

Diagnosis	n (%)
HCC	7 (8)
Hemangioma	49 (51)
Calcified granuloma	8 (8)
Liver cyst	8 (8)
Regenerate nodule	3 (3)
Arteriovenous shunt	6 (6)
Normal liver parenchyma	15 (19)

HCC: Hepatocellular carcinoma.

which 18816 participants were randomly allocated to a screening or control group; the proportion of small HCC was higher at 45.3% vs 0%, with resection rates in the screening group of 46.5% vs 7.5%. Mortality was also reduced by 37%. Our studies showed that resection was possible in 80% of the screened HCC cases, while a tumor size of < 5 cm was found in 70%. Despite a large tumor size in some patients in this study, there were also other alternative treatment modalities such as TACE or portal vein embolization which could convert initially unresectable HCC to a resectable tumor^[23]. However, there is no established preoperative adjuvant strategy to improve prognosis in HCC patients^[24]. Cirrhosis is another important factor determining operability because it indicates poor hepatic reserve. The heterogeneous proportion of cirrhosis in screened populations in several studies seemed to affect the treatment modality^[10,25]. Liver transplantation is the only potentially curative method for both chronic liver disease and HCC. Nevertheless, low availability of liver transplantation facilities in many parts of the world and scarcity of donor organs, with long and unpredictable waiting times, are the main obstacles to the long-term survival of most HCC patients. None of the HCC patients in our study received liver transplantation.

There are conflicting results regarding the benefit and efficacy of screening for improved survival. A randomized controlled study conducted in Qidong, China^[8] resulted in an earlier diagnosis of liver cancer, but no reduction in mortality. Mok *et al*^[10] studied HCC patients with a tumor size of < 5 cm detected using ultrasonography; serum AFP levels revealed no benefit of early detection. In contrast, according to our findings the 3-year survival rate of HCC patients was 90%. This rate was as high as the rate in stage 0 and

stage A HCC based on BCLC staging^[26].

According to many international guidelines and reports^[17,18,27], AUS is the most appropriate tool for screening and surveillance. In the current study, AUS could detect almost all HCC lesions with a sensitivity and specificity of 94% and 82%, respectively, and more efficiently than using serum AFP level. Increased AFP levels were observed in only half of the HCC patients, most of whom had a tumor size of > 5 cm. This was supported by a report demonstrating that increased AFP levels might be indicative of severe liver injury^[28], and AFP can be increased by many other factors. AFP levels may be elevated in patients without HCC, especially during exacerbation of the hepatitis. Another study using AFP for HCC detection achieved the same diagnostic accuracy as in our study^[14]. Thus, serum AFP alone is not an ideal marker for the screening of HCC. The combined use of AFP and ultrasonography is also not recommended because of increased false positive rates that lead to increased costs.

Based on cost-effectiveness data, several guidelines recommend that the HCC screening in patients with chronic HBV begins when they are cirrhotic, or for non-cirrhotic patients at the age of 40 and 50 years for Asian males and females, respectively; this is because many CHB infection patients in Asian countries develop early onset HCC at a younger age^[29]. The strength of our study is the fact that we also screened a large number of CHB infection patients aged < 40 years (half of the participants). However, none of the younger patients developed early onset HCC as of the last follow-up. Thus, our data support a low incidence of HCC in the younger age group who may further require the use of other clinical or laboratory predictors to determine the risk of early-onset HCC.

Patient adherence can play an important role in cancer screening and surveillance. Wong *et al.*^[30] reported an association between HCC screening adherence and greater access to curative treatment. In our study, the adherence rate was very high during the 42 mo of follow-up. Our strategic methods included short public service messages, reminder phone calls and letters, appointments at the most convenient time for the patient, and home visits. After implementing this strategy, the adherence rate improved from 52% to 99.9% in later visits (unpublished data). Lastly, the present data support our previous economic evaluation and budget impact analysis^[31]. Semi-annual AUS was a cost-effective option for HCC screening and surveillance in CHB patients aged 40-50 years, at a willingness-to-pay threshold of 160000 Thai Baht per quality adjusted life years.

Our study is the first large cohort study to report the high prevalence and incidence rates of HCC in CHB patients in Thailand through a screening and surveillance program involving AUS. AUS examination was more sensitive than serum AFP testing for early-stage HCC detection. Serum AFP did not provide any

benefit for HCC screening and surveillance. The active AUS screening program led to a high 3-year survival benefit in our CHB population who developed HCC. It is suggested that a screening and surveillance program be implemented in CHB patients aged ≥ 40 years, at a national policy level, to improve HCC detection at a potentially operable and curable stage.

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COMMENTS

Background

Hepatitis B virus (HBV)-associated hepatocellular carcinoma (HCC) is a major health problem in all regions of Thailand. Abdominal ultrasonography (AUS) and serum alpha-fetoprotein (AFP) measurement are widely accepted as routine HCC screening and surveillance tests in chronic hepatitis B (CHB) patients to detect early-stage HCC; however, there are conflicting results regarding survival improvement. At present, no national policy for screening and early detection of HCC is currently available in Thailand. The study was designed to evaluate a screening and surveillance program for naïve CHB patients using AUS and serum AFP for early-stage HCC detection and its potential to improve survival after HCC diagnosis.

Research frontiers

This study confirmed the high prevalence and incidence rates of HCC in Thailand. Semi-annual examination using AUS can detect early stage HCC; this led to a high 3-year survival benefit. Serum AFP did not add benefit for HCC screening and surveillance.

Innovations and breakthroughs

The current study suggests the benefit of AUS as an early-stage HCC screening and surveillance tool in treatment-naïve CHB patients aged ≥ 40 years.

Applications

This study provides additional evidence supporting the role of HCC screening and surveillance using AUS. The results should change the way the policy makers in developing countries, in particular Thailand, perceive and deal with CHB cases; they hopefully will lead to the implementation of an effective screening and surveillance program regarding HCC in CHB patients residing in an endemic area.

Peer-review

This is an excellent work with very useful clinical information on the value of AUS screening of HBV-infected patients for early detection of HCC in Thailand.

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Cost effectiveness of routine duodenal biopsies in iron deficiency anemia

Efrat Broide, Shay Matalon, Ofra Kriger-Sharabi, Vered Richter, Haim Shirin, Moshe Leshno

Efrat Broide, Shay Matalon, Ofra Kriger-Sharabi, Vered Richter, Haim Shirin, The Kamila Gonczarowski Institute of Gastroenterology, Assaf Harofeh Medical Center, Zerifin 70300, Israel

Moshe Leshno, Faculty of Business Administration, Tel-Aviv University, Tel-Aviv 39040, Israel

Author contributions: Broide E and Matalon S contributed equally to this work; Broide E is the guarantor of article; Leshno M, Broide E and Matalon S performed the research, collected and analyzed the data; Shirin H and Leshno M designed the research study; Broide E, Matalon S, Richter V and Kriger-Sharabi O wrote the paper; Matalon S, Richter V and Kriger-Sharabi O contributed to the design of the study.

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Correspondence to: Efrat Broide, MD, Professor of Pediatrics Gastroenterology, The Kamila Gonczarowski Institute of Gastroenterology, Assaf Harofeh Medical Center, Beer Ya'akov, Zerifin 70300, Israel. efbroide@yahoo.com
Telephone: +972-54419077
Fax: +972-89779727

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Abstract

AIM

To investigate the cost effectiveness of routine small bowel biopsies (SBBs) in patients with iron deficiency anemia (IDA) independent of their celiac disease (CD) serology test results.

METHODS

We used a state transition Markov model. Two strategies were compared: routine SBBs during esophagogastroduodenoscopy (EGD) in all patients with IDA regardless their celiac serology status (strategy A) vs SBBs only in IDA patients with positive serology (strategy B). The main outcomes were quality adjusted life years (QALY), average cost and the incremental cost effectiveness ratio (ICER). One way sensitivity analysis was performed on all variables and two way sensitivity analysis on selected variables were done. In order to validate the results, a Monte Carlo simulation of 100 sample trials with 10, and an acceptability curve were performed.

RESULTS

Strategy A of routine SBBs yielded 19.888 QALYs with a cost of \$218.10 compared to 19.887 QALYs and \$234.17 in strategy B. In terms of cost-effectiveness, strategy A was the dominant strategy, as long as the cost of SBBs stayed less than \$67. In addition, the ICER of strategy A was preferable, providing the cost of biopsy stays under \$77. Monte Carlo simulation demonstrated that strategy A yielded the same QALY but with lower costs than strategy B.

CONCLUSION

Our model suggests that EGD with routine SBBs is a cost-effective approach with improved QALYs in patients

with IDA when the prevalence of CD is 5% or greater. SBBs should be a routine screening tool for CD among patients with IDA, regardless of their celiac antibody status.

Key words: Celiac disease; Iron deficiency anemia; Cost-effectiveness; Esophagogastroduodenoscopy; Markov model

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Core tip: Common practice, and current recommendations are to perform celiac serology tests and endoscopies to iron deficiency patients. Obtaining duodenal biopsies is usually reserved to patients with positive celiac serology. We aimed to investigate the cost effectiveness of routine duodenal biopsies in each and every patient with iron deficiency anemia, regardless serology status. We found this strategy to have a higher quality adjusted life years, a lower cost and a higher incremental cost effectiveness ratio over the common strategy of selected duodenal biopsies strategy.

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INTRODUCTION

Iron deficiency anemia (IDA) is a common presentation of celiac disease (CD) found in as many as 50% of the patients at the time of diagnosis^[1-4]. However, the need for routine duodenal biopsies in IDA patients, independent of their celiac serology results, is still debated^[5-8].

In 2000, The British Society of Gastroenterology guidelines recommended that in the absence of overt blood loss or any other obvious cause for IDA, all patients with IDA should undergo esophagogastroduodenoscopy (EGD), including obtaining small bowel biopsies (SBBs)^[9]. A decade later, the revised guidelines published by the same group, recommended that in IDA patients SBBs should be obtained only if celiac serology was positive or not performed^[10]. This recommendation is based on the estimation that for every 330 biopsies taken from patients with negative celiac serology, one patient with CD would be diagnosed, meaning an additional cost of £35000 (in prices of 2011)^[10]. No guidelines exist in the literature regarding screening for CD among those with IDA in the United States.

On the other hand, ACG clinical guidelines for the

diagnosis and management of CD published in 2013^[11] recommend routine SBBs during EGD in populations where the probability for CD is 5% or more. As the prevalence of CD among patients with IDA is about 5%, duodenal biopsies and serology for tTG antibodies are therefore recommended^[11]. In addition, the recently published guidelines regarding diagnosis and management of CD recommend that individuals who undergo EGD due to anemia, weight loss or diarrhea should have SBBs, irrespective of their serology status^[2].

In the present study we compared two strategies in patients with IDA; routine SBBs during EGD in all patients regardless their celiac serology status (strategy A) vs SBBs only in patients with positive serology (strategy B). The main outcomes were quality adjusted life years (QALY), average cost and the incremental cost effectiveness ratio (ICER). In addition, we also looked at life expectancy (LE) and ran an acceptability model for our results.

MATERIALS AND METHODS

Model

We constructed a state transition Markov model^[12,13] to evaluate whether there is an added benefit in terms of QALYs and ICER, as well as whether it is more cost-effective to perform routinely SBBs in patients with IDA. QALY is the most common outcome unit used in cost-effectiveness analysis and it incorporates both the quality of life and life expectancy, while ICER is a statistic used in cost-effectiveness analysis to summarize the cost-effectiveness of a health care intervention. We compared two strategies: routine SBBs in all patients, referred to EGD due to IDA (strategy A); or screening all patients with IDA for CD serology and SBBs only in those patients with positive serology (strategy B). Three possibilities were analyzed: IDA patients without CD; IDA patients who have CD but were misdiagnosed due to negative serological test, and IDA patients correctly diagnosed with CD. Patients were placed into one of the following health states in each cycle of the model; (1) No CD; (2) CD but undiagnosed (*i.e.*, considered healthy); (3) potential CD, defined as positive serology for CD but without villous atrophy (normal mucosa, Marsh 1 or 2)^[14]; (4) CD under normal diet; (5) CD under strict gluten free diet (GFD); and (6) Death.

We assumed that the gold standard for the diagnosis of CD is SBBs, therefore in patients who have normal SBBs, CD can be ruled out.

Prevalence of CD in patients with IDA

IDA presents in 80%-90% of patients with CD and has been described as the sole presentation of the disease even in the absence of gastrointestinal symptoms^[15,16]. The prevalence of CD among IDA patients varies between 1.3%-14.6% in different countries^[17-19]. We

estimated a prevalence of 5% of CD in IDA with a range of 5%-10%.

Assumptions regarding diagnostic tests

In a systematic review of 42 studies published by Lewis *et al.*^[20] the pooled sensitivity and specificity of IgA tTG antibody test was 92.8% (95%CI: 91.9-93.6), and 98.1% (95%CI: 97.8-98.4) respectively. According to the National Institute of Clinical Excellence (NICE), using biopsy results as a reference (Marsh 3 only as positive diagnosis of CD), tTG antibody test had a sensitivity of 87.5% (95%CI: 66.5-96.7), and specificity of 89.5% (95%CI: 87.0-91.6)^[21]. tTG antibody test has been recommended to be the first step in screening for CD, as it is less costly than IgA anti endomysial antibodies, and has similar diagnostic value for the diagnosis and/or exclusion of CD^[22,23]. In patients with IgA deficiency, serology based on IgG (IgG deamidated gliadin peptide) should be taken.

For the sensitivity analysis, we selected these base-case values according to those reported by Lewis *et al.*^[22] (sensitivity value of 93% with a range of 89%-96%, and specificity of 99% with a range of 89%-99%).

Assumption regarding the hazard ratio for mortality

During 45 years of follow-up, all-cause mortality was greater in persons with undiagnosed CD compared to those who were sero-negative for CD, with a HR of 3.9 (95%CI: 2.0-7.5), $P < 0.01$ ^[24]. Cottone *et al.*^[25] reported a 3.8 standardized mortality ratio (SMR) rate in patients with CD compared to the general population and this increased risk seemed to be due to non-Hodgkin's lymphoma. Corrao *et al.*^[26] documented increased SMRs of 2.5 (1.3-4.6) among patients with a diagnostic delay of 120 mo or more; of 2.9 (1.8-4.3) among patients with severe CD; and of 5.2 (3.4-7.8) among patients with poor adherence to a GFD^[26]. West and others found that the overall HR for mortality in CD was 2.09 in the first year after diagnosis and declined to 1.1 after the first year of diagnosis^[27]. Meta-analysis done by Tio *et al.*^[28] showed an increase risk for all-cause mortality in CD patients with an OR of 1.24 (95%CI: 1.19-1.3). On the contrary, other studies reported no excess in overall mortality in patients with undetected CD compared to the general population^[29,30]. We used an HR of 1.39 in the base case with a range of 1.33-1.45.

Adherence rate to a gluten free diet

There are different definitions regarding adherence to gluten free diet (GFD), all intrinsically linked to the manner in which adherence was assessed and measured. The rate of a GFD in CD patients for over a period of 10 years was reported to be between 50%-80% while the rate of strict adherence to GFD ranges between 42%-91%^[31,32]. We assumed that the annual transition probability from adherence to GFD to

a normal diet is estimated to be 0.9 (range of 0.6-0.9), and that the transition probability from strict diet to normal diet is estimated to be 0.2 (range 0.2-0.6).

Transition probability from potential CD to mucosal flattening

Biagi *et al.*^[33] published the cumulative probability of mucosal flattening in patients with potential CD over a period of 24 mo. In order to extrapolate the transition probability for a longer time we assumed that cumulative incidence of mucosal flattening follows Weibull distribution. The parameters of the Weibull distribution were estimated using Nelder-Mead Algorithm to optimize the model parameters. For the Weibull distribution, the transition probability is given by: $1 - \text{StSt-}u = 1 - \exp[-\lambda t - u \gamma \exp(\lambda t \gamma)]$. Where u is the cycle time, λ is the scale parameter and γ is the shape parameter of the Weibull distribution.

Assumptions regarding utilities

Many CD patients suffer from persistent clinical symptoms and reduced health-related quality of life despite a strict GFD^[34-36]. A Swedish study documented that patients with CD who had been under a GFD for 10 years, with variable adherence rate, had a calculated utility of 0.717 compared with an average of 0.726 for the general population^[37]. We used in our sensitivity analysis, a utility value of 0.92 (range of 0.90-1) for newly diagnosed CD patients, and value of 0.99 (range 0.95-1) for CD patients after GFD. Since the aim of identifying CD in patients with IDA is to improve quality of life, we projected gains in QALYs and costs for the remainder of patients' lives. This was accomplished by calculating remaining life expectancy as a function of age from the United States Life Tables^[37].

Costs

We calculated costs of serology (tTG Antibody test only), EGD, SBBs and the cost of evaluating symptoms. The average payments for each coded procedure were based on the 2013 Medicare Fee Schedule (<http://gi.org/wp-content/uploads/2013/02/2013ACGPayment-Grid.pdf>) (Table 1). According to expert's opinion we assumed that about 50% (range of 30%-80%) of the patients with a missed diagnosis of CD, will need a repeat investigation. We considered the cost of such an investigation to be \$150. The calculations neglected the cost of EGD complications, as the risk of perforation, duodenal hematoma or death is very rare.

Assumption regarding the percentage of undiagnosed CD who will undergo further investigation after one year

According to expert's opinion, we assume that 50% of all patients with a missed diagnosis of CD (biopsies were not performed during the first EGD) will need further investigation within the next coming year. In the sensitivity analysis we used a value of 50%,

Table 1 Variables costs

Variable	Base	Low	High
Time horizon	Life-time	70	100
Age of patient, yr	45	40	60
Cost of Biopsy (including complication)	60	60	80
Cost of tTG Ab's test	70	60	80
Cost of evaluating symptoms	150	100	300
Cost of upper endoscopy	350	300	400
Increasing rate of cost of patient with undiagnosed CD	1.25	1	1.4

All costs are in 2013 (USD).

Table 2 Base-case values and ranges used in sensitivity analysis in our model

Variable	Base	Low	High	Ref.
Transition probability continuing strict diet given the patient was on strict diet ¹	0.90	0.60	0.90	[37,38]
Transition probability to normal diet from strict diet	0.20	0.20	0.60	Assumption
Transition probability continuing normal diet given the patient was on normal diet	0.30	0.20	0.60	Assumption
Probability finding celiac due to symptoms	0.20	0.10	0.40	Assumption
Prevalence of celiac in IDA patient	0.05	0.05	0.10	[18,20-23]
Discount rate of costs	0.03	0.00	0.05	Assumption
Specificity of serologic tests	0.99	0.89	0.99	[24-26]
Sensitivity of serologic tests	0.93	0.89	0.96	[24-26]
Utility of CD	0.92	0.90	1.00	[40-43]
Utility of GFD	0.99	0.95	1.00	Assumption
Rate of Overt CD	0.80	0.75	0.90	Assumption
of Weibull distribution	0.2686	0.26	0.29	Assumption
of Weibull distribution	1.1668	1.15	1.18	Assumption
HR mortality of CD patients vs general population	1.39	1.33	1.45	[29-34]

¹Annual transitions probabilities. IDA: Iron deficiency anemia; CD: Celiac disease; GFD: Gluten free diet; HR: Hazard ratio.

ranging from 30% to 80%.

Sensitivity analyses

We performed one-way sensitivity analyses on all variables (transition probabilities, costs, and utilities) in the model, and two-way sensitivity analysis on selected variables. One-way sensitivity analyses were performed in order to identify variables that had an impact on the decision of which strategy is the dominant one. In addition, we conducted a Monte Carlo simulation of 100 sampling trials with 10000 patients. Each variable was tested separately to determine whether varying the particular variable over a broad range alters the ICER. In the base line analysis, we assumed a willingness-to-pay (WTP) threshold of \$50000 per QALY as being cost-effective^[38]. Table 2 summarizes the base-case values

and ranges used in sensitivity analysis in our model.

RESULTS

QALYs outcome

The strategy to obtain SBBs regardless the serological results (strategy A) yielded 19.888 QALY, and dominated the strategy of conducting SBBs only in patients with a positive serological test (strategy B), which had 19.887 QALY. Figure 1A is a Tornado plot showing the different parameters that had an impact on the incremental QALY. The most influential parameters on the QALY outcome were the prevalence of CD in IDA patients, the utility of CD and the probability of identifying CD due to symptoms. As demonstrated in the one-way sensitivity analysis figures of these parameters (Figure 1B-D, respectively), the dominance of the strategy of performing SBBs in all IDA patients was robust.

Cost outcome

The average cost of strategy A was \$218.10 vs \$234.17 for strategy B. These results are explained by the lower cost of performing serological tests only in patients with positive CD biopsies compared to performing them in the whole study population. As shown in Figure 2A, the costs of serological tests, biopsies and patients' symptoms evaluation had the greatest impact on the incremental average cost.

We next tested the two-way sensitivity analysis with the costs of serological tests and SBBs. As shown in Figure 2B, as long as the cost of SBBs is less than \$67, strategy A dominates strategy B. These results are independent of the costs of serological test within the range of \$60-\$80. However, when the cost of SBBs is greater than \$67, the dominant strategy depends on both the cost of SBBs and the cost of the serological tests.

ICER

Analyzing the ICER for both strategies, we found that strategy A was more cost effective than strategy B. Figure 3A demonstrates the parameters that affected the ICER analysis. As shown in Figure 3B, for a cost of biopsy less than \$77, the universal SBBs strategy dominated (*i.e.*, costs less with higher QALY). However, when the cost of biopsy was greater than \$77, the ICER increased up to almost \$3000 per QALY and the strategy of conducting SBBs only in patients with positive serology dominated.

The results of the Monte Carlo simulation (Figure 4A) of 100 sampling trials with 10000 patients in each trial, demonstrate that strategy A yielded the same QALY with lower costs than strategy B. Finally, in order to verify our results, we performed an acceptability curve (Figure 4B). This figure demonstrates that as the willingness to pay for each QALY increases, the validity of our cost effectiveness study increases as well. For

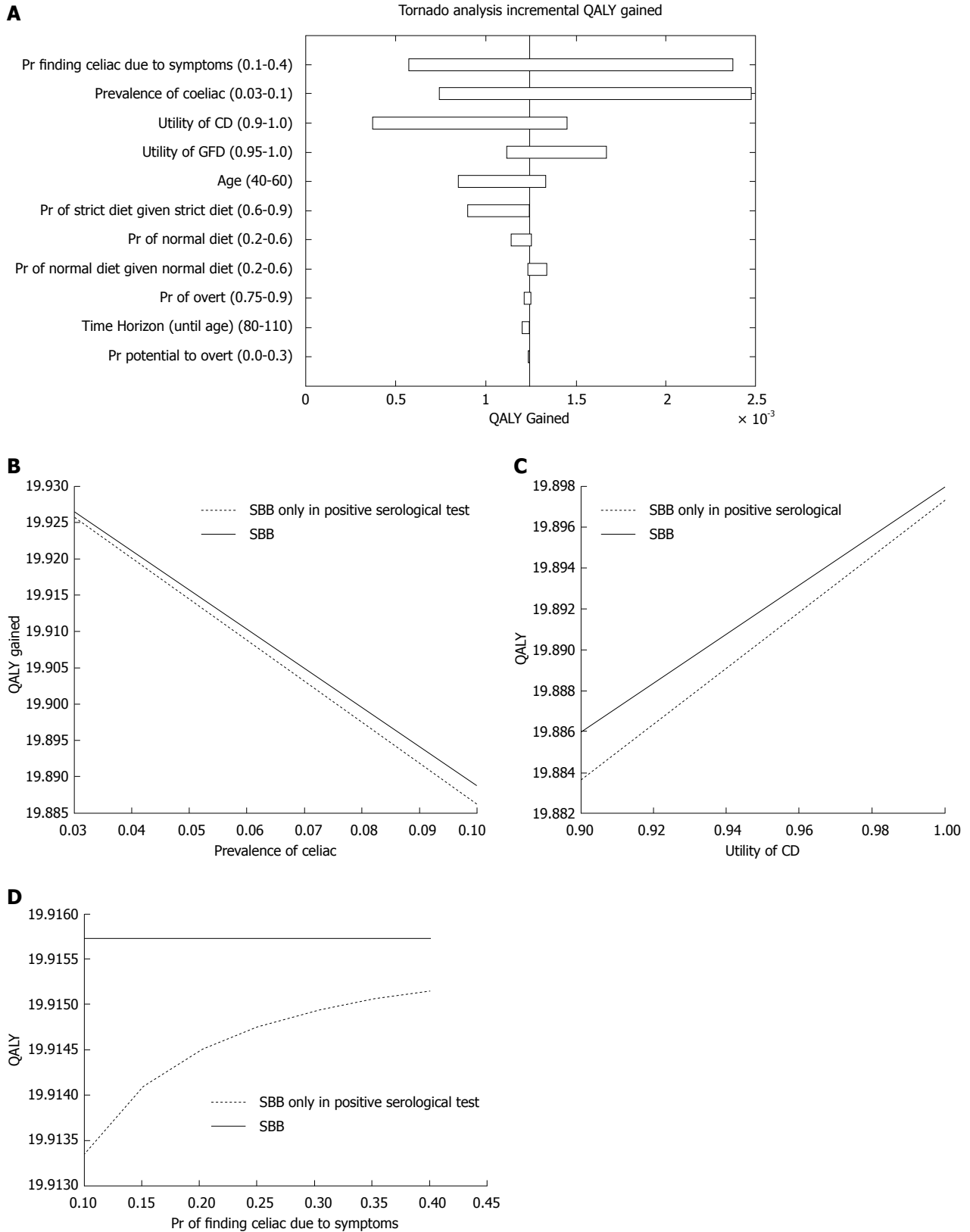


Figure 1 Quality adjusted life-years outcome. A: Influential parameters on the incremental quality adjusted life-years (QALY); B: One-way sensitivity analysis figure of the prevalence of celiac disease; C: One-way sensitivity analysis figure of the utility of celiac disease; D: One-way sensitivity analysis figure of the probability of diagnosing celiac disease due to symptoms. CD: Celiac disease; GFD: Gluten free diet; SBB: Small bowel biopsy.

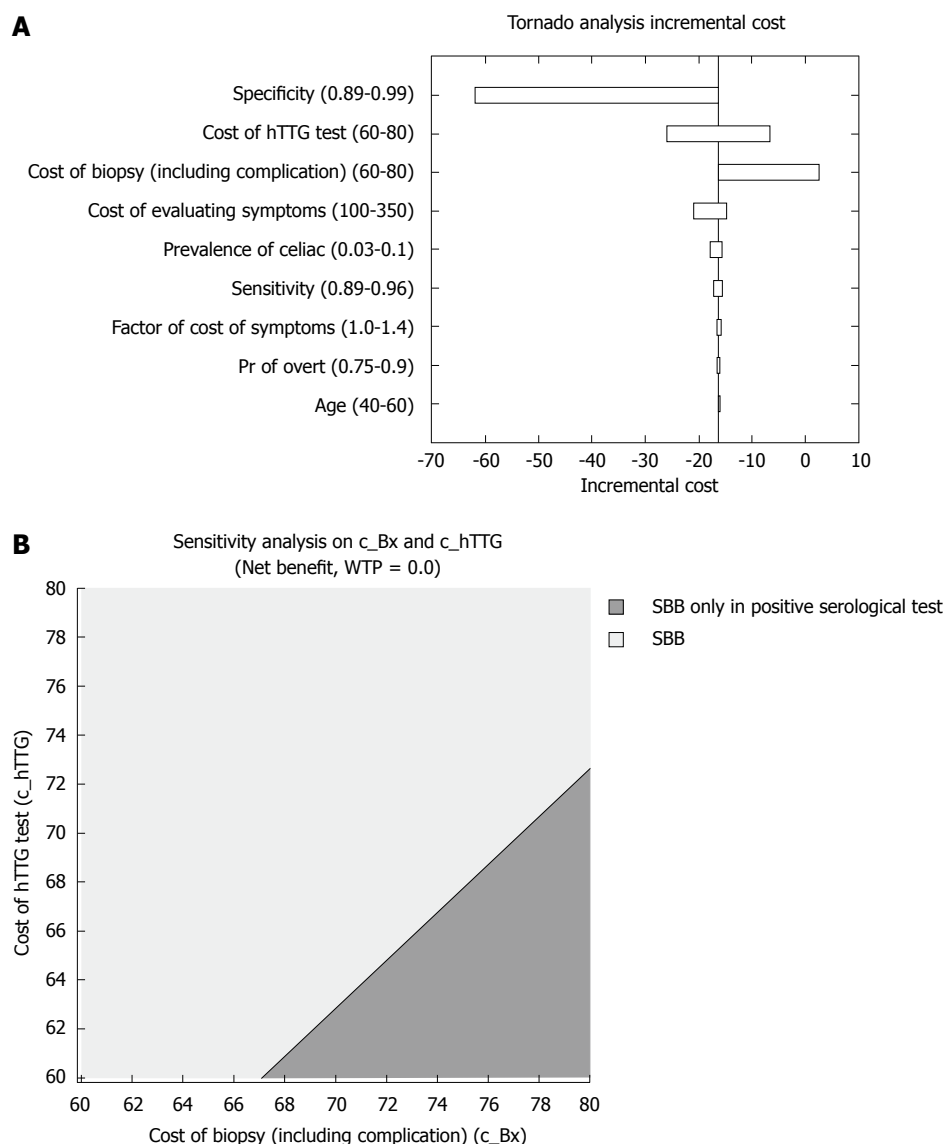


Figure 2 Cost outcome. A: Influential parameters on the incremental cost; B: Two-way sensitivity analysis depicting the less costly strategy, in regards to serological tests and small bowel biopsies prices.

example, if the willingness to pay for 1 QALY is \$10000 the probability of the validity of our results would be approximately 98%.

DISCUSSION

The results of our model provide additional data that performing duodenal biopsies in patients with IDA, regardless of their celiac serology status or even in patients with IDA and negative serology, is a cost effective strategy with improved QALY. This strategy substantially dominated the strategy of performing SBBs only in patients with positive celiac serology.

We applied our model to patients aged ≥ 45 years, but routine duodenal biopsy for a diagnosis of CD was found to increase diagnostic yield even in patients over 65 years of age. (1) The role of performing routine endoscopic duodenal biopsies during the evaluation of IDA is increasingly emphasized, as the prevalence

of CD in this population was reported to increase the diagnostic yield by 5%-14%^[3,15]. There are several known macroscopic endoscopic markers for CD including: scalloping, mosaicism, fissuring and others^[39]. As normal endoscopic looking mucosa does not exclude the diagnosis of CD, there are several new technology development of endoscopic tools and procedures proved to increase the yield of the diagnosis. These tools include: narrow band imaging, optical coherence tomography, water immersion technique, i-scan technology and confocal laser endomicroscopy^[39-43]. However according to the published clinical guidelines of CD^[11] SBB is still required for the diagnosis. Moreover, endoscopic lesions that may explain IDA should not preclude obtaining SBBs^[2].

Recent studies have indicated that patients with untreated CD have significant morbidity and even severe complications^[44]. These patients have a decreased quality of life, as the stigma of chronic disorder

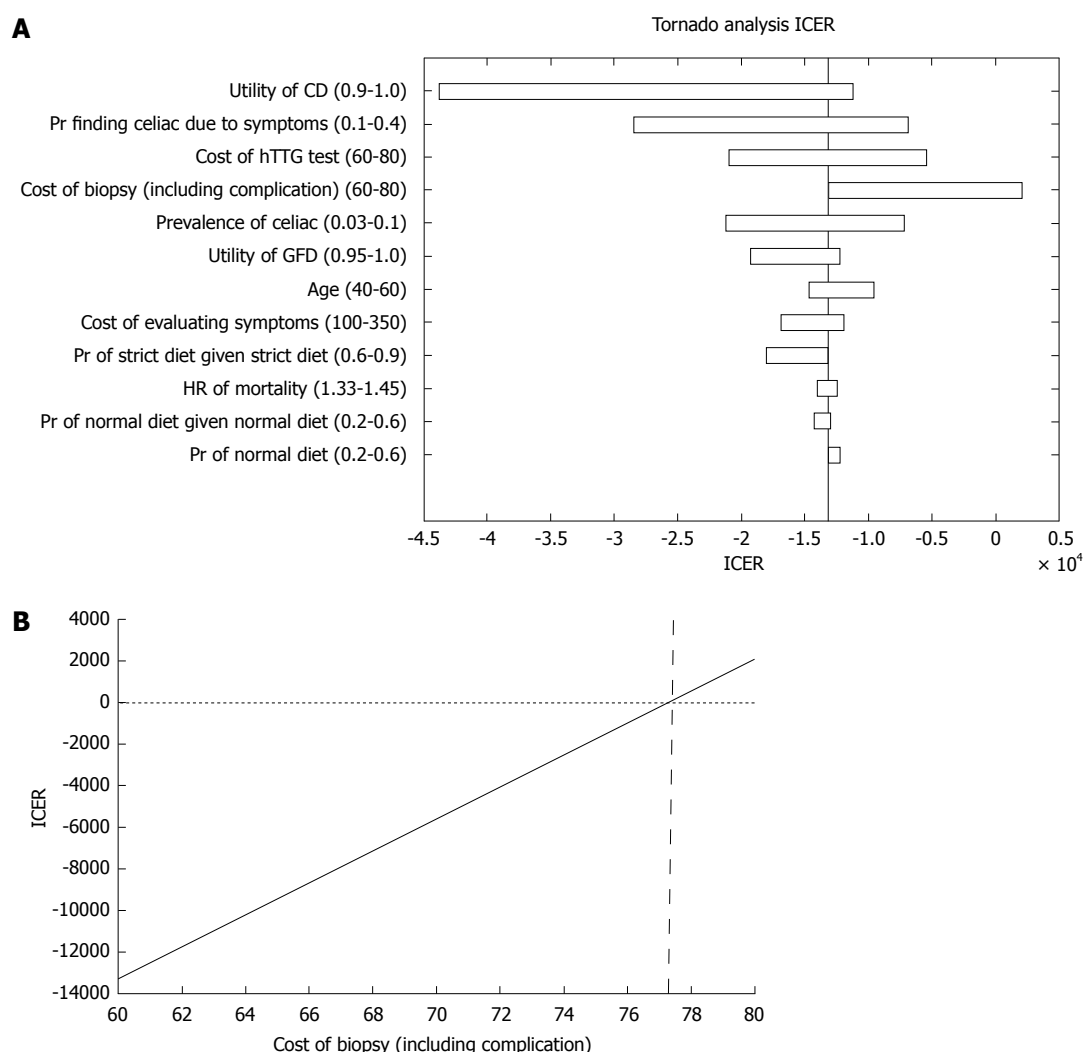


Figure 3 Incremental cost-effectiveness ratio outcome. A: Parameters that affected the incremental cost-effectiveness ratio (ICER) analysis; B: ICER of both strategies in regard to the cost of small bowel biopsies. CD: Celiac disease; GFD: Gluten free diet.

and the need for major dietary restrictions, increase the self-perceived burden of their illness^[45]. As a result, in some studies, even well treated CD patients have failed to attain well-being compare to that of the general population^[46,47]. The mean pre-treatment QALY score for the CD population in the literature was 0.66 and increased to 0.86 after initiating a GFD. A lower QALY score was associated with a delay over 2 years since the appearance of symptoms to diagnosis^[48]. In our study QALY had a major impact on the final conclusion to perform SBBs in all IDA patients.

We also demonstrated that strategy A had a better ICER (less total costs with more QALY) suggesting that conducting SBBs as a first step in all IDA patients followed by tTG serology in patients with \geq Marsh 3a^[11], would be a more cost effective strategy. However, not all CD patients have positive serology and lesser degree of villous atrophy is more frequently seen in sero-negative patients^[49]. As such, there is a difficulty to identify this group of patients unless the physician maintains a high degree of clinical suspicion. Therefore, strategy B, of performing SBBs only in

patients with positive serology may lead to under-diagnosis of CD^[50,51].

Finally, we also measured life expectancy (LE) without a discount rate, for the two strategies (data not shown). We found that for a 45 old patient with IDA, strategy A had a longer LE compared to strategy B, 78.04843 years vs 78.04770 years respectively. The prevalence of CD in IDA patients was found to be the parameter with the greatest impact on LE.

Our study has several limitations: we compared conducting SBBs unrelated to serological results assuming that the patients did not have a serological test, which reduced the total cost. However, in real practice, most patients would have had a serologic test done before facing the question whether or not to conduct an EGD with SBBs. Consequently testing the same outcomes, we applied our model, to a strategy of conducting SBBs in patients with IDA and negative serology. As Goddard *et al*^[10] estimated, if the pretest probability of CD in patients presenting only with IDA is 5%, the post-test probability of CD is 0.3%. This means there is a need of duodenal biopsies from

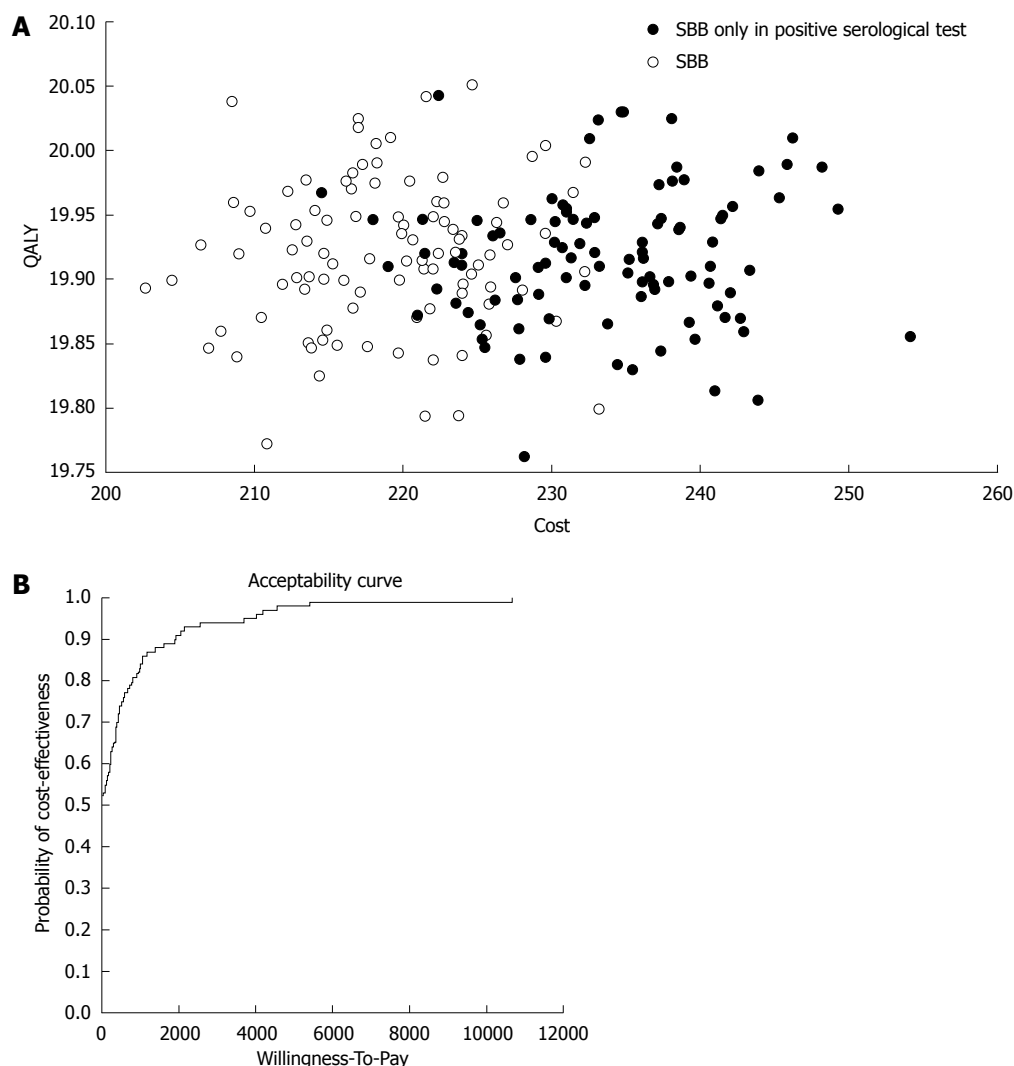


Figure 4 Validation. A: Monte Carlo simulation of 100 sample trials with 10000 patients in each trial; B: Acceptability curve that validate the cost effectiveness results of the study in relation to the willingness to pay for each quality adjusted life year (QALY). SBB: Small bowel biopsy.

about 330 tTG antibody-negative patients to detect one extra patient with CD at an estimated additional cost of £35000 (about \$50000). If the cost of SBBs is \$60, the additional cost would be \$19800. Under the assumptions of our model, the average cost of "No SBBs" strategy was \$83.1 compared to \$141.1 in the "SBBs" strategy (additional of \$57.98). The average QALY in the "No SBBs" strategy was 19.4399 vs 19.4412 QALY in the "SBBs" strategy. The ICER was \$45393 per QALY, which is still considered cost-effective ($< \$50000$)^[38]. However, when the cost of SBBs is higher than \$60 the ICER increases and yield a non-cost-effective ICER. Since the cost of SBBs in our model was lower compared to the analysis performed by Goddard *et al.*^[10] we challenged our model by calculating SBBs with a higher cost, ranging between \$50 to \$120 (Figure 5A). Under these costs the ICER ranges between \$37000 to \$95000 per QALY. Our results revealed that as long as the utility of CD is less than 0.92, the ICER would still be under \$50000 for 1 QALY (Figure 5B).

Our model was mainly prevalence dependent, and was not dependent solely on the sensitivity and specificity values of tTG antibodies. Therefore, we ran the model twice: first we used the values of sensitivity and specificity reported by the NICE 2012: 87% (95%CI: 65.3-96.6), and 96.9% (95%CI: 95.3-98.0) respectively. In the second run, we used the values used by the British Society of Gastroenterology guidelines for the management of IDA^[10]. Analyzing both the Tornado plot and the sensitivity analysis in both cases showed stable results and proved that performing SBBs in IDA patients, regardless the tTG serology results, was still the dominant strategy. This model may miss potential CD patients, who will not be further evaluated for CD serology, due to normal histological findings. In these cases we presume that if IDA will be refractory and persistent serology for CD will ultimately be checked.

In conclusion our model suggests that, EGD with SBBs appears to be a cost-effective approach

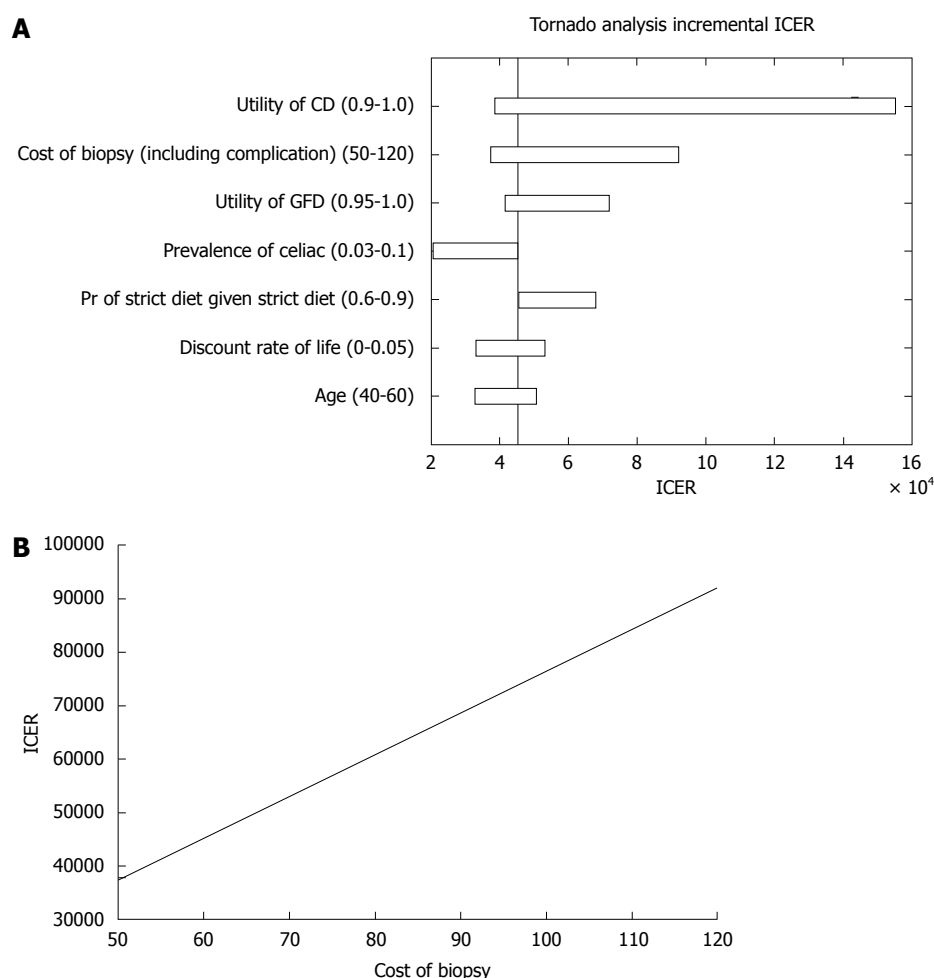


Figure 5 Incremental cost-effectiveness ratio outcome in patients with negative celiac serology. A: Parameters that affected the incremental cost-effectiveness ratio (ICER) analysis; B: A one way sensitivity analysis of ICER in regards to the cost of small bowel biopsies. CD: Celiac disease; GFD: Gluten free diet.

with improved QALYs in patients with IDA when the prevalence of CD is 5% or greater. SBBs should be a routine screening tool for CD among patients with IDA, regardless of their celiac antibody status.

COMMENTS

Background

Iron deficiency anemia (IDA) is a common presentation of celiac disease (CD) found in as many as 50% of the patients at the time of diagnosis. However, the need for routine duodenal biopsies in IDA patients, independent of their celiac serology results, is still debated. The latest clinical guidelines for the diagnosis and management of CD published in 2013 recommend routine SBBs during upper endoscopy in when the probability for CD is 5% or more. As the prevalence of CD among patients with IDA is about 5%, duodenal biopsies and serology for tTG antibodies are therefore recommended.

Research frontiers

According to recent studies, quality of life of celiac patients is inferior compared to healthy population. About 5% of IDA patients are diagnosed with CD. No studies were done to estimate the cost effectiveness of routine SBBs, regardless celiac serology status, in IDA patients in order to diagnose CD patients earlier and by that reduce morbidity and mortality. This is the first study which explore the cost effectiveness of performing routine SBBs to diagnose celiac in IDA patients. The authors measured, using a Markov model, quality adjusted life years (QALY), average cost and the incremental cost effectiveness

ratio (ICER).

Innovations and breakthroughs

This model shows that routine SBBs, regardless of serology status, yielded higher QALY, lower cost and higher ICER than performing SBBs only in patients with positive serology. These results were valid as long as cost of SBBs stayed less than \$67. In addition, the ICER of strategy A was preferable, providing the cost of biopsy stays under \$77.

Applications

Upper endoscopy with routine SBBs is a cost-effective approach with improved QALYs in patients with IDA when the prevalence of CD is 5% or greater. SBBs should be a routine screening tool for CD among patients with IDA, regardless of their celiac antibody status.

Terminology

QALY - The most common outcome unit used in cost-effectiveness analysis is QALY, which incorporates both the quality of life and life expectancy. The quality of each health state is measured on a scale of 0 to 1 and is based on patient's preferences over the health states. ICER - The incremental cost-effectiveness ratio is a statistic used in cost-effectiveness analysis to summarise the cost-effectiveness of a health care intervention, defined by the difference in cost between two possible interventions, divided by the difference in their effect.

Peer-review

The manuscript is well written, and the idea is interesting, as it shows a novel

methodological approach to the management of celiac disease, with relevant saving of money.

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Global epidemiology of hepatitis C virus infection: An up-date of the distribution and circulation of hepatitis C virus genotypes

Arnolfo Petruzzello, Samantha Marigliano, Giovanna Loquercio, Anna Cozzolino, Carmela Cacciapuoti

Arnolfo Petruzzello, Samantha Marigliano, Giovanna Loquercio, Anna Cozzolino, Carmela Cacciapuoti, Laboratory of Virology and Molecular Biology "V. Tridente", IRCCS Italia, Fondazione "G. Pascale", 80131 Naples, Italy

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Correspondence to: Arnolfo Petruzzello, PhD, Laboratory of Virology and Molecular Biology "V. Tridente", IRCCS Italia, Fondazione "G. Pascale", Via Mariano Semmola, 80131 Naples, Italy. a.petruzzello@istitutotumori.na.it
 Telephone: +39-81-5903433
 Fax: +39-81-5453854

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Abstract

AIM

To review Hepatitis C virus (HCV) prevalence and genotypes distribution worldwide.

METHODS

We conducted a systematic study which represents one of the most comprehensive effort to quantify global HCV epidemiology, using the best available published data between 2000 and 2015 from 138 countries (about 90% of the global population), grouped in 20 geographical areas (with the exclusion of Oceania), as defined by the Global Burden of Diseases project (GBD). Countries for which we were unable to obtain HCV genotype prevalence data were excluded from calculations of regional proportions, although their populations were included in the total population size of each region when generating regional genotype prevalence estimates.

RESULTS

Total global HCV prevalence is estimated at 2.5% (177.5 million of HCV infected adults), ranging from 2.9% in Africa and 1.3% in Americas, with a global viraemic rate of 67% (118.9 million of HCV RNA positive cases), varying from 64.4% in Asia to 74.8% in Australasia. HCV genotype 1 is the most prevalent worldwide (49.1%), followed by genotype 3 (17.9%), 4 (16.8%) and 2 (11.0%). Genotypes 5 and 6 are responsible for the remaining < 5%. While genotypes 1 and 3 are common worldwide, the largest proportion of genotypes 4 and 5 is in lower-income countries. Although HCV genotypes 1 and 3 infections are the most prevalent globally (67.0% if considered together), other genotypes are found more commonly in lower-

income countries where still account for a significant proportion of HCV cases.

CONCLUSION

A more precise knowledge of HCV genotype distribution will be helpful to best inform national healthcare models to improve access to new treatments.

Key words: Hepatitis C virus genotype; Epidemiology; Hepatitis C virus; Hepatitis C virus prevalence; Hepatitis C virus infections; Viraemia

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Core tip: Hepatitis C virus (HCV) infection is a global public health burden, causing an increasing level of liver-related morbidity and mortality due to the disease progression. Unfortunately, in many countries, there is a lack of robust epidemiological data, especially HCV genotypes distribution, upon which to base country-specific prevention, diagnosis and treatment strategies in order to reduce the disease burden represented by HCV. Stratification by viral genotypes at national and regional level, and a better understanding of viral diversity within target populations, might also critically inform the rational design and testing of future HCV vaccines.

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INTRODUCTION

Hepatitis C virus (HCV) is one of the major globally cause of death and morbidity^[1] and recent estimates showed an increase in its seroprevalence over the last decade to 2.8%, corresponding to > 185 million infections worldwide^[2].

Chronic HCV infection is often associated with the development of liver cirrhosis, hepatocellular cancer, liver failure, and death^[3] especially in HIV-positive patients during active antiretroviral therapy^[4]. It has been estimated that while the incidence of HCV infection seems to decrease in the developed world, mortality secondary related to HCV infection will continue to increase over the next 20 years^[5]. So, although many data suggest that HCV infection could be eliminated in the next 15-20 years with focused therapeutic strategies^[6,7], a good understanding of HCV infections should be required to develop strategies to prevent new infections.

Previous and more recent studies have reported

regional prevalence estimates, but always considering a limited number of countries^[6,8-13]. A more recent analysis, instead, estimates a global HCV prevalence, but provides only regional estimates^[2]. In all the cases, studies focused only on the presence of HCV antibodies generally overestimate the disease burden because they include also patients healed spontaneously or through treatments. So, although antibodies to HCV (anti-HCV) are at present the most commonly available marker of HCV infection, used both to estimate its prevalence and to compare HCV infection levels globally, the most important indicator of HCV diffusion seems to be its classification into different genetic variants. At present, in fact, the length of the therapy and the opportunity to associate interferon and/or ribavirin with the new direct-acting antiviral (DAA) therapies still remain partially dependent on HCV genotype. A detailed understanding of the regional HCV genotype distribution might led to the development of specific national treatment strategies.

Up to now, HCV is classified into seven recognized genotypes^[1-5,14-16] on the basis of sequence of the viral genome^[17], each differing at 30%-35% of nucleotide sites and into 67 confirmed and 20 provisional subtypes, differing at < 15% of nucleotide sites^[18].

There are several methods used to determine HCV genotypes, all using the direct sequencing of specific polymerase chain reaction (PCR)-amplified portions of the virus (NS5, core, E1 and 5' UTR regions)^[18-21], often in combination with the phylogenetic analysis^[17]. Apart from the restriction fragment length polymorphisms, in which restriction enzymes are able to recognize genotype-specific cleavage sites in a PCR-amplified DNA fragments^[22] but whose sensitivity and specificity seems to need further investigations^[23] and Kinetic amplification^[24,25], whose reproducibility will need surely more investigations in the future, the most common method for its simple interpretation is surely line probe assays (LiPA)^[14-16], in which PCR amplified fragments are able to hybridize to genotype-specific probes immobilized on nitrocellulose strips.

Genotype-specific antibodies able to recognize the NS4 region of HCV have also been exploited^[20], even if this type of serological genotyping still lacks specificity and sensitivity with huge limits of its clinical applications.

The geographic distribution of HCV genotypes is rather complex. The so called "epidemic subtypes" - specifically 1a, 1b, 2a, and 3a - are widely distributed worldwide and account for a great proportion of the totality of HCV cases, especially in high income countries. They were probably spread in the 70's and 80's, before HCV sequencing, through transfusion, blood products and drug abusers^[26-29].

The so called "endemic" strains, instead, are comparatively more rare and have been restricted for long time in specific regions, as West Africa, Southern Asia, Central Africa and South Eastern Asia^[26,30,31]. At present, only one genotype 7 infection has been

reported from a Central African immigrant in Canada^[32].

The present global distribution of HCV genotypes has undoubtedly been influenced by historical events (for example the trans-Atlantic slave trade) or by the contemporary human migration trends^[33].

Since the duration and the cost of clinical treatments useful to fight HCV infection are still mostly impacted by the different clinical evolution that each HCV subtype seems to have, especially until pan-genotypic therapies will not be able to reach the global market, a correct knowledge of the HCV distribution is surely crucial to contain this global burden disease. At the present, however, more than half of the countries in the world do not have robust studies of the HCV infected population.

The purpose of this study was to conduct a comprehensive review of recently published literature to estimate anti-HCV prevalence, the viraemic rate (HCV-RNA positive) and genotype distribution to generate a global estimate of HCV disease burden.

MATERIALS AND METHODS

A comprehensive review of the literature from 2000 to 2015 was used to gather country-specific data on prevalence, number of diagnosed individuals and genotype distribution. References were identified through two sources: indexed journals and non-indexed sources. Indexed articles were found by searching PubMed and regional databases using the following terms: "[Country Name] and [hepatitis c or HCV] and [prevalence]" or "[genotypes] or [viraemia]". Furthermore, references cited within the articles were used.

Regions included in the analysis were those defined by the Global Burden of Diseases, Injuries, and Risk Factors 2010 (GBD) study^[34,35]. This study defined 21 regions that were "epidemiologically homogenous as possible so that information from detailed studies in one country can plausibly be extrapolated to other countries in the region to create burden estimates that are useful to individual countries in planning for health sector activities"^[36].

The average HCV prevalence for each continent was calculated by dividing the sum of data reported from each region to the total number of countries within the region. The first- and second generation immuno-assay tests which usually provide false-positive results overestimating the total infected population^[37,38] were not used to estimate the country's HCV prevalence, selecting only studies in which it was used a third generation test. Similarly, the genotyping and the viraemic rate was obtained only by considering studies in which LiPA test and a well described PCR RT system (range of sensitivity and linearity) was used.

Article titles and abstracts were reviewed for relevance and the following data were extracted from full articles or abstracts: anti-HCV prevalence, viraemic prevalence, viraemic rate and genotypes distribution.

The infected general population was composed of high-risk groups [e.g., people who inject drugs (PWID's), dialysis patients, haemophiliacs, minority ethnic groups, etc.] as well as non-high-risk groups that contracted the disease through contact with infected blood (e.g. nosocomial infections, dental procedures, etc.). Studies with a sample size of less than 1000 and studies published prior to 2000 or not in English were excluded from the analysis.

Five hundred and fifty-seven articles were selected based on relevance. In addition, non-indexed sources were identified through searches of individual country's Ministry of Health's websites and international health agency reports. If articles contained the same patient cohort then this cohort was only counted once.

Because the first- and second generation immuno-assay tests may provide false-positive results, which can overestimate the total infected population, care was taken to use only studies that used the latest generation tests to estimate the country's prevalence.

In the majority of studies HCV cases were classified by LiPA Method at the genotype level, but not at the subtype level, so we decided to use only genotype classification using as general method that proposed by Simmonds *et al.*^[17]. In case of one or more genotypes identified in the same patient, we classified it as "mixed". We did not include genotype 7 in the analysis.

Countries for which we were unable to obtain HCV genotype prevalence data were excluded from calculations of regional proportions, although their populations were included in the total population size of each region when generating regional genotype prevalence estimates.

RESULTS

Africa

The GBD subdivides the African continent into 4 macro areas: Central, East, West and Southern, whereas the Saharan area (North Africa) is generally associated with Middle East countries.

The estimated prevalence of anti-HCV in the whole Sub-Saharan Africa is 2.9% with an estimated 26.9 million of cases (Table 1), ranging from 6.0% in the Central area and 0.9% in the Southern countries (Table 2).

The viraemic rate is estimated at 70.5%, with a peak of 79.6% in the Western Africa (Table 2), accounting almost 20.0 millions of active HCV replication cases (Table 1).

The most representative genotype is the genotype 4 (G4) (28.1%), followed by genotypes 1 (G1) (26.3%), genotype 2 (G2) (23.7%), genotype 5 (G5) (12.2%), and genotype 3 (G3) (6.3%). No cases of genotype 6 (G6) and only a small percentage of mixed genotypes were reported (Table 3). The genotype distribution shows high variability among the four macro-areas studied, ranging between 82.9% (Central Africa) and 0.6% (West Africa) for G4, 35.7% (Southern Africa)

Table 1 Global anti-hepatitis C virus prevalence and number of infected individuals (all ages)

Continent	Anti- HCV prevalence (%)	Viraemic rate (%)	2013 population (millions)	Anti- HCV infected (millions)	Viraemic HCV infected (millions)
Africa	2.9	70.5	927.0	26.9	19.0
North Africa/Middle East	2.7	68.8	469.0	12.7	8.7
America	1.3	74.0	953.7	12.4	9.2
Asia	2.8	64.4	3985.0	111.6	71.9
Australasia	1.8	74.8	28.0	0.5	0.4
Europe	1.8	72.4	742.5	13.4	9.7
Total	2.5	67.0	7105.2	177.5	118.9

HCV: Hepatitis C virus.

Table 2 Regional estimates of hepatitis C virus seroprevalence and viraemia

Regions	Anti-HCV prevalence (%)	Viraemic rate (%)
Central Sub-Saharan Africa	6.0	68.5
EastSub-Saharan Africa	2.4	65.0
Southern Sub-Saharan Africa	0.9	69.0
WestSub-Saharan Africa	2.4	79.6
North Africa and Middle East	2.7	68.8
North America, High Income	1.2	75.7
Caribbean	1.5	70.0
Andean Latin America	1.2	70.0
Central Latin America	1.4	75.8
Southern Latin America	1.5	79.5
Tropical Latin America	1.6	80.2
Central Asia	5.8	48.7
East Asia	2.8	63.6
Pacific Asia, High-income	1.1	70.5
South Asia	2.5	78.5
Southeast Asia	1.6	60.5
Australasia	1.8	74.8
Europe, Central	1.3	76.6
Europe, Eastern	3.1	69.6
Europe, Western	0.9	71.0

HCV: Hepatitis C virus.

and 0% (West and Central Africa) for G5, 62.9% (West Africa) and 1.2% (Southern Africa) for G2 and 7.4 (East Africa) and 0.8% (Central Africa) for G3. No high variability was observed for G1 (36.2%, 31.4% and 25.5% in East, Southern and West Africa respectively), except for Central Africa (12.3%) (Table 4).

Central Sub-Saharan Africa: The countries studied in this area are Burundi, Cameroon, Central African Republic (CAR), Chad, Congo, Democratic Republic of Congo (DRC), Equatorial Guinea, Gabon, Rwanda, Sudan and Uganda.

The prevalence of HCV in the general population is 6.0%, ranging between 1.7% and 13.8%, depending on the country, with an average viraemic rate estimated at 68.5% (Table 2). The countries with the highest prevalence include Cameroon (13.8%), Burundi (11.3%) and Gabon (9.2%). The countries with the lowest prevalence Equatorial Guinea (1.7%), CAR (2.4%) and Sudan (2.8%) (Figure 1A).

The predominant genotype is G4 (82.9%), followed by G1 (12.3%), G2 (4.0%) and G3 (0.8%). No cases of G5 and G6 were reported (Table 4). In some countries, like DRC and Gabon, G4 is quite the only genotype observed (96.8% and 91.9%, respectively), whereas in others, as Equatorial Guinea, it has been detected an increasing percentage of G1 (35.0%) (Figure 1B). No genotypes distribution data are available from Burundi, Cameroon, Chad, Congo, Rwanda, Sudan and Uganda

East Sub-Saharan Africa: Analysis of this area includes studies from Eritrea, Ethiopia, Kenya, Madagascar, Mozambique, Somalia and Tanzania and shows an average prevalence of HCV infection of 2.4%, ranging between 0.9% and 3.2%, depending on the country, and the lowest viraemic rate of the whole African continent (65.0%) (Table 2). The countries with the highest prevalence are Tanzania (3.2%) and Mozambique (2.8%), those with the lowest Kenya (0.9%) and Somalia (1.5%) (Figure 1A).

The predominant genotype in this area is G1 (36.2%), followed by G2 (26.8%), G4 (16.6%), G5 (13.0%) and G3 (7.4%). No cases of G6 are reported (Table 4). Except for Ethiopia, where G4 represents about the half of all the genotypes described and Madagascar, where the only two genotypes found are G1 (52.9%) and G4 (47.1%), in other countries all the genotypes are equally present (Figure 1B). No genotypes distribution data are available from Eritrea, Kenya, Somalia and Tanzania.

Southern Sub-Saharan Africa: Data concerning this macro area are reported from Malawi, South Africa, Swaziland, Zambia and Zimbabwe and show an average HCV prevalence of 0.9% and a viraemic rate estimated at 69.0% (Table 2). The countries with the highest prevalence are Zimbabwe (2.0%) and Swaziland (1.5%), whereas South Africa and Zambia show the lowest prevalence (0.1% and 0.2%, respectively) (Figure 1A).

The more common genotype is the G5 (35.7%), as reported from the only country where genotypes prevalence has been studied (South Africa), followed by G1 (31.4%), G3 (12.6%) and G4 (12.4%). No cases of G6 were reported and only a small percentage

Table 3 Global prevalence of hepatitis C virus genotypes

Continents	G1 (%)	G2 (%)	G3 (%)	G4 (%)	G5 (%)	G6 (%)	Mixed
Africa	26.3	23.7	6.3	28.1	12.2	-	3.4
North Africa/Middle East	27.3	0.8	6.3	65.3	0.3	-	-
America	74.5	10.2	10.6	1.7	0.1	0.3	2.6
Asia	46.6	18.6	22.4	1.0	0.1	7.0	4.3
Australasia	55.0	6.5	36.0	1.2	-	1.3	-
Europe	64.4	5.5	25.5	3.7	0.1	0.1	0.7
Total (excludes Oceania)	49.1	11.0	17.9	16.8	2.0	1.4	1.8

Table 4 Regional estimates of hepatitis C virus genotypes

Regions	G1 (%)	G2 (%)	G3 (%)	G4 (%)	G5 (%)	G6 (%)	Mixed
Central Sub-Saharan Africa	12.3	4.0	0.8	82.9	-	-	-
East Sub-Saharan Africa	36.2	26.8	7.4	16.6	13.0	-	-
Southern Sub-Saharan Africa	31.4	1.2	12.6	12.4	35.7	-	6.7
West Sub-Saharan Africa	25.5	62.9	4.4	0.6	-	-	6.6
North Africa and Middle East	27.3	0.8	6.3	65.3	0.3	-	-
North America	66.3	13.1	15.7	4.3	-	0.6	-
Caribbean	83.0	7.2	2.1	0.6	-	0.1	7.0
Andean Latin America	86.0	2.0	10.0	-	-	-	2.0
Central Latin America	74.6	21.6	3.3	0.1	0.1	-	0.3
Southern Latin America	72.0	13.3	13.5	0.9	0.1	0.1	0.1
Tropical Latin America	64.8	4.6	30.2	0.2	0.1	-	0.1
Central Asia	70.4	8.6	19.6	-	-	-	1.4
East Asia	53.5	31.7	5.4	0.1	-	3.3	6.0
Pacific Asia, High-Income	58.7	39.7	0.4	0.1	-	0.5	0.6
South Asia	15.5	1.9	66.7	3.7	0.1	0.5	11.6
Southeast Asia	35.2	11.1	19.9	0.9	0.4	30.8	1.7
Australasia	55.0	6.5	36.0	1.2	-	1.3	-
Central Europe	70.0	3.2	21.0	4.9	-	0.1	0.8
Eastern Europe	68.1	4.3	26.6	0.5	-	-	0.5
Western Europe	55.1	8.9	29.0	5.8	0.2	0.1	0.8

of G2 is described (Figure 1B). No genotypes distribution data are available from Malawi, Swaziland, Zambia and Zimbabwe.

West Sub-Saharan Africa: The countries studied in this area are Benin, Burkina Faso, Cote d'Ivoire, Gambia, Ghana, Guinea Bissau, Mauritania, Niger, Nigeria, Senegal and Togo. The prevalence of HCV is 2.4%, ranging between 1.1% and 5.5%, depending on the country, with the higher viraemic rate of all the African continent (79.6%) (Table 2). The countries with the highest prevalence include Guinea Bissau (5.5%) and Burkina Faso (4.9%), those with the lowest Mauritania (1.1%) and Benin (1.6%) (Figure 1A).

The predominant genotype is G2 (62.9%), followed by G1 (25.5%) and G3 (4.4%) (Table 4). No cases of G5 and G6 were reported and only a small percentage of G4 was described from Burkina Faso. The genotype distribution shows a great heterogeneity from country to country. If G2 accounts nearly the totality of genotyped cases from Guinea Bissau (98.2%) and Ghana (87.0%), Nigeria shows a prevalent circulation of G1 (85.0%) (Figure 1B). No genotypes distribution data are available from Benin, Cote d'Ivoire, Mauritania, Niger, Senegal and Togo.

North Africa/Middle East

This region includes Algeria, Egypt, Iran, Iraq, Israel, Jordan, Kuwait, Lebanon, Libya, Morocco, Oman, Palestine, Qatar, Saudi Arabia, Syria, Tunisia, Turkey, United Arab Emirates and Yemen with an estimated prevalence of HCV in the general population of 2.7%, corresponding to an estimated 12.7 million of cases. The HCV viraemic rate is estimated at 68.8%, accounting over 8 millions of active HCV replication cases (Table 1). No adult HCV prevalence and/or viraemic data are available from Jordan, Kuwait, Lebanon, Oman, Palestine, Syria and United Arab Emirates. The countries with the highest prevalence include, over Egypt (14.7%), Iraq (3.2%) and Yemen (2.2%). The countries with the lowest Qatar (0.9%) and Turkey (1.0%) (Figure 2A).

The predominant genotype is the genotype 4 (65.3%), followed by 1 (27.3%), 3 (6.3%). Only small percentages of genotype 2 and 5 and no genotype 6 were reported (Table 3).

In this area genotypes distribution is highly heterogenic. If genotype 4 accounts almost the totality of genotyped cases from Egypt (93.1%) and over the half of the total cases in Iraq, Kuwait, Palestine, Saudi Arabia and Syria (52.9%, 54.2%, 64.1%, 60.0% and 59.0%), Turkey, Tunisia, Jordan and Morocco show a

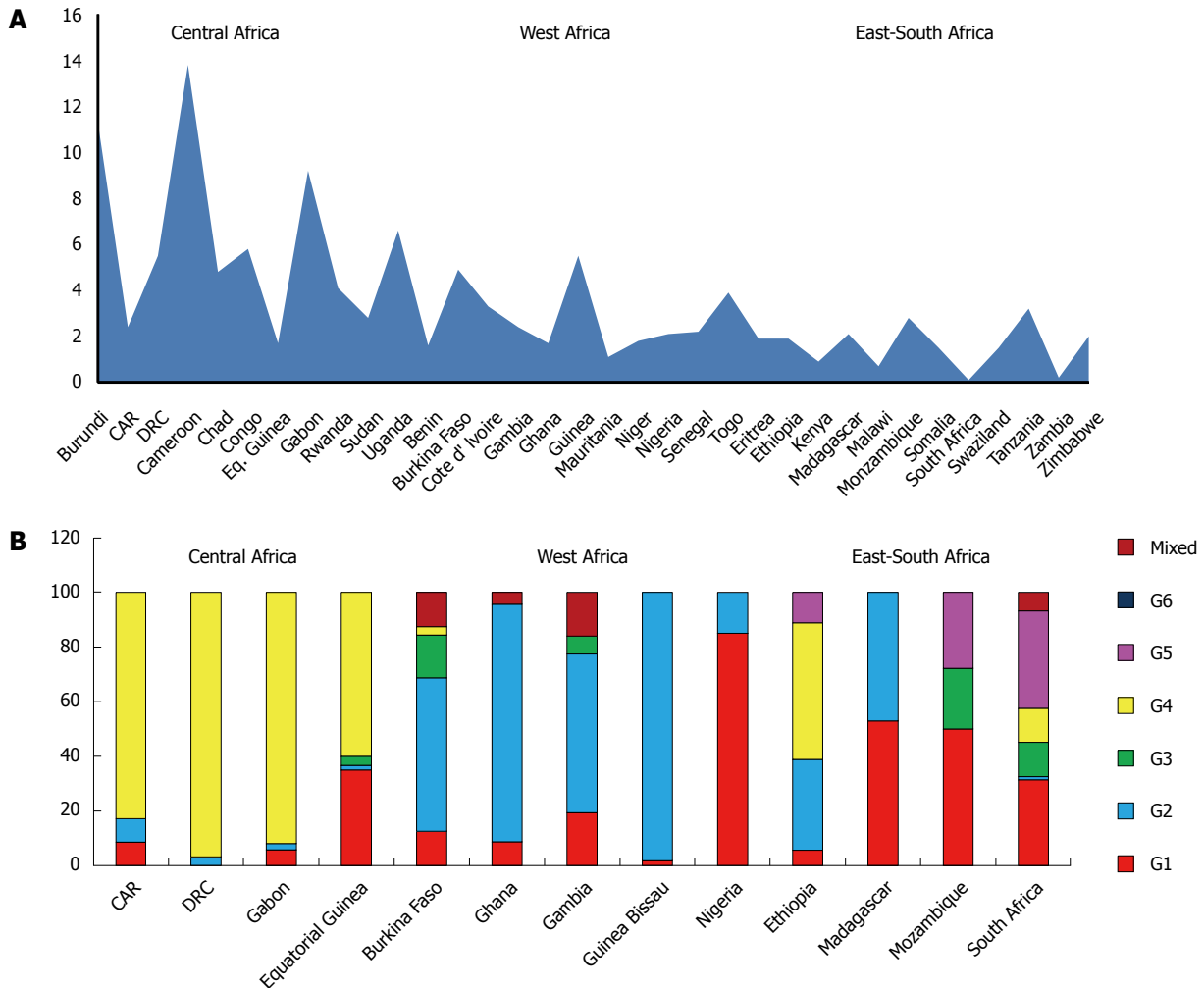


Figure 1 Anti-hepatitis C virus prevalence (A) and genotype distribution (B) in Sub-Saharan Africa.

prevalent circulation of genotype 1 (91.8%, 84.0%, 77.3% and 75.9%, respectively). The majority of cases of genotype 3 are described in Iran and United Arab Emirates (27.5% and 23.8%), whereas in Libya all the genotypes are equally distributed. No data of genotypes distribution are available from Israel, Oman, Qatar, and Yemen (Figure 2B).

Americas

The GBD subdivides the American continent in three macro areas: North America, Caribbean and Latin America.

The estimated prevalence of HCV in the whole continent is 1.3%, with more than 12 million of estimated cases, while the viraemic rate is 74.0%, accounting over 9 million of cases with active HCV replication (Table 1).

The most representative genotype is G1 (74.5%), followed by G3 (10.6%), G2 (10.2%) and G4 (1.7%). Only small percentages of G5, G6 and not well classified are reported (Table 3). Genotype distribution shows high variability among the three macro-areas studied, ranging between 83.0% of the Caribbean area and 66.3% in Northern America for G1, 21.6% (Central

Latin America) and 2.0% (Andean Latin America) for G2, 15.7% (Northern America) and 2.1% (Caribbean) for G3 (Table 4).

North America, High Income: The countries studied in this area are Canada and United States, with an average prevalence of HCV in the general population of 1.2%, ranging between 1.1% in Canada and 1.3% in the United States, and a viraemic rate estimated at 75.7% (Figure 3A).

The predominant genotype is G1 (66.3%), followed by G3 (15.7%), G2 (13.1%) and G4 (4.3%). No cases of G5 and only a small percentage of G6 has been reported (Table 4). In both the two studied countries, G1 represents over the half of all genotypes observed (60.0% and 72.5%, respectively), whereas G3 is more frequent in Canada (22.3%), if compared to the United States (8.9%) (Figure 3B).

Caribbean: In this area, studies responding to the selected parameters are available only from Cuba, Dominican Republic and Puerto Rico.

The prevalence of HCV is 1.5%, ranging between 0.8% in Cuba and 2.3% in Puerto Rico (Figure 3A),

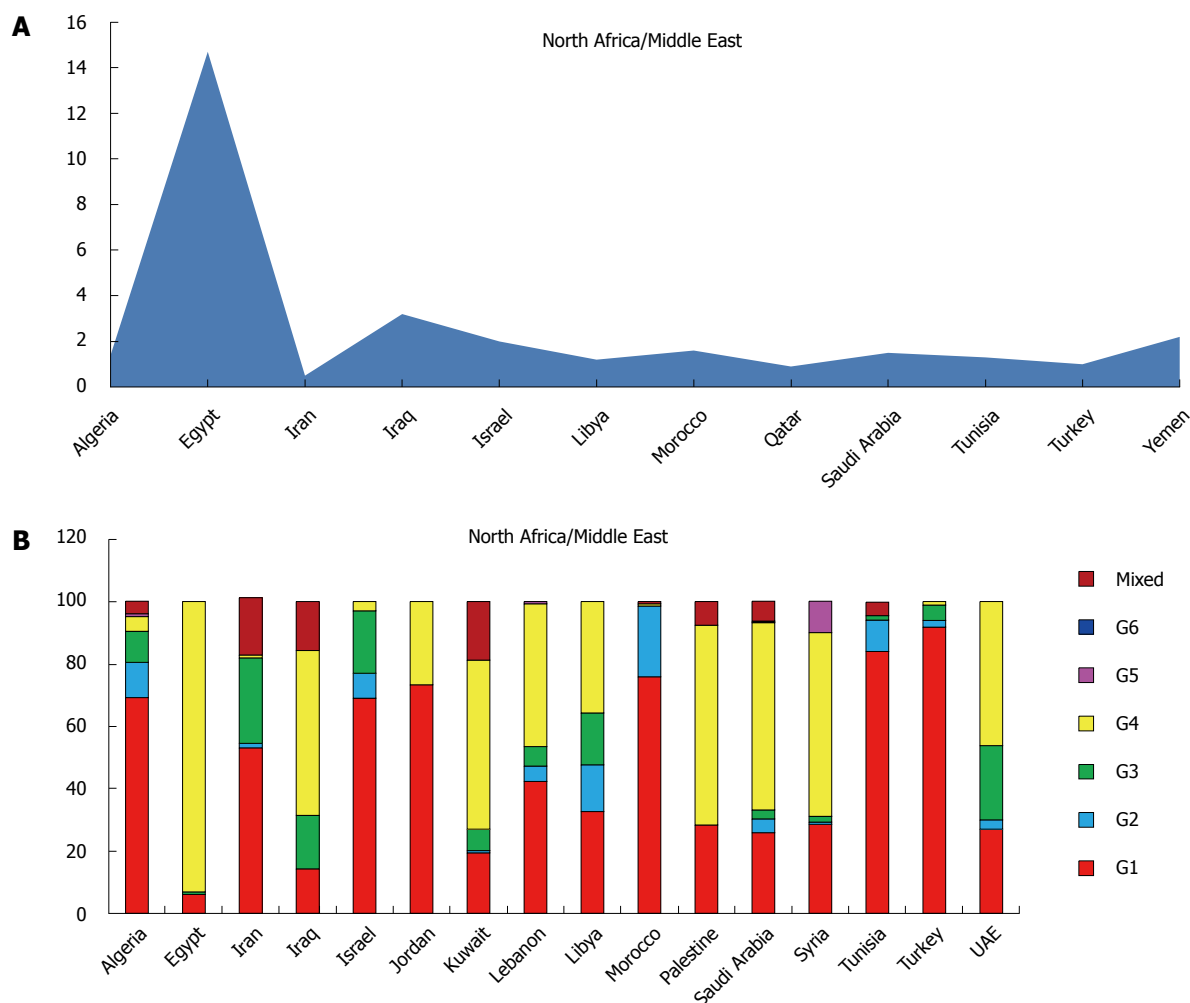


Figure 2 Anti-hepatitis C virus prevalence (A) and genotype distribution (B) in North Africa/Middle Eastern Area.

with a viraemic rate estimated at 70.0% (Table 2). No adult HCV prevalence and/or viraemic data are available from Cuba and Dominican Republic.

The predominant genotype in this area is G1 (83.0%), followed by G2 (7.2%), G3 (2.1%). No cases of G5 and only a small percentage of G4 and G6 are reported (Table 4). In all the studied countries (Cuba, Dominican Republic and Puerto Rico), G1 is almost the only observed (98.0%, 62.6% and 82.1%, respectively) (Figure 3B).

Latin America: GBD sub divides Latin America in four different areas: Andean, which includes Bolivia and Peru, Central, formed by Colombia, Mexico and Venezuela, Southern, represented by Argentina, Chile and Uruguay and Tropical that includes only Brazil.

The HCV prevalence is 1.4%, ranging between 1.2% in the Andean area and 1.6% in the Tropical zone, with a viraemic rate of 76.4% (Table 2).

All the countries in the 4 macro areas show almost the same HCV prevalence: Brazil (1.6%), Argentina and Venezuela (1.5%), Mexico (1.4%) and Peru (1.2%) (Figure 3A). No adult HCV prevalence and/or viraemic data are available from Colombia and Chile.

The predominant genotype is G1 (74.3%), followed by G3 (14.2%), G2 (10.4%). Only a small percentage of G4, G5, G6 and mixed genotypes are reported (Table 4). In some of the countries studied (Peru, Colombia and Chile) G1 is almost the only observed (86.0%, 88.5% and 80.6%, respectively), whereas in others (Venezuela, Mexico and Argentina) G2 shows a significantly percentage (34.4%, 21.8% and 24.7%). Brazil is the only one country where G3 has a high percentage (30.2%). No genotypes distribution data are available from Bolivia and Uruguay (Figure 3B).

Asia

GBD subdivides Asia in five macro areas: Pacific, Central, East, South, and Southeast.

The estimated prevalence of HCV in the whole Asian continent is 2.8%, accounting over 60% of the estimated cases worldwide. The HCV viraemic rate is 64.4%, with 71.9 million of active HCV replication cases (Table 1).

The predominant genotype is the 1 (46.6%), followed by G3 (22.4%), G2 (18.6%) and G6 (7.0%). Only small percentages of G4, G5 and mixed or not further classified genotypes are reported (Table 3).

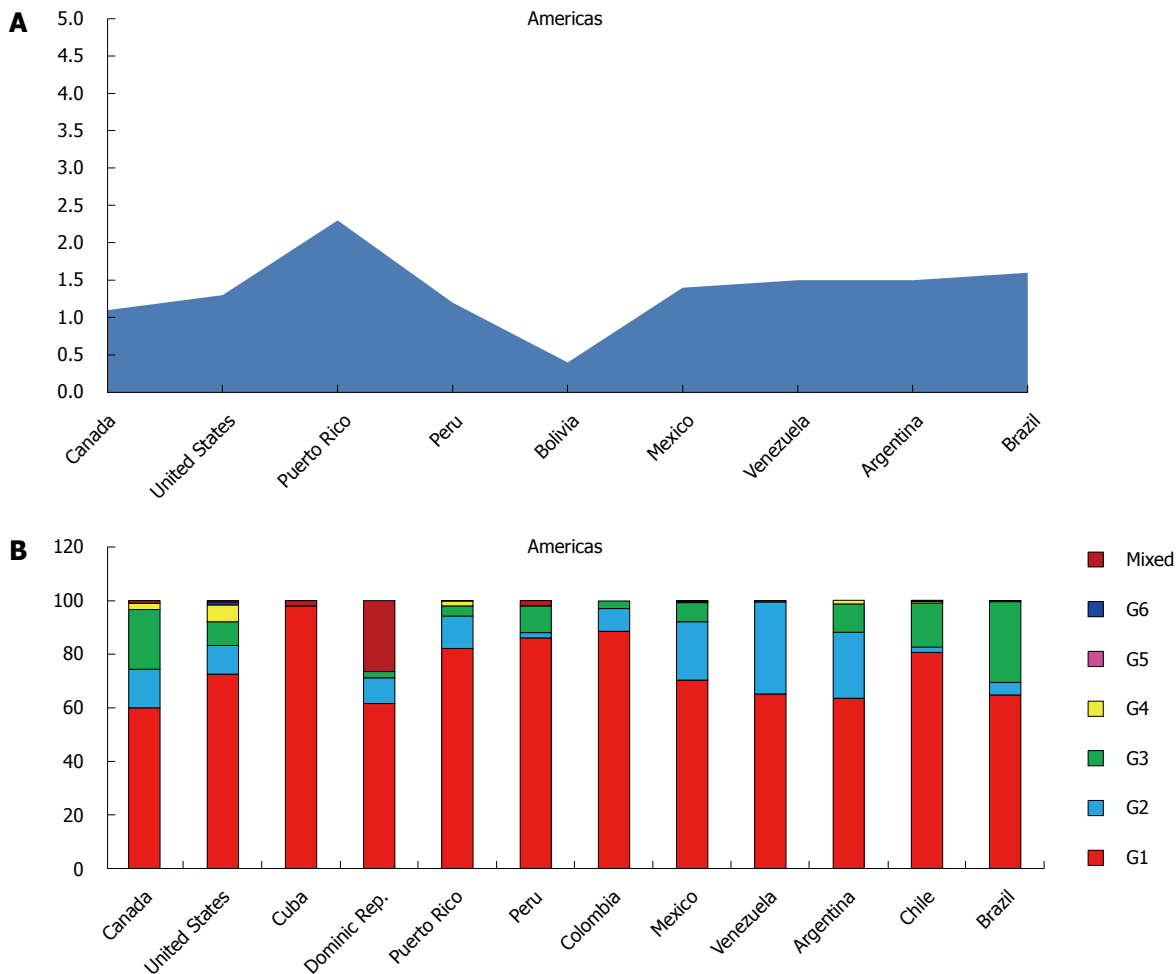


Figure 3 Anti-hepatitis C virus prevalence (A) and genotype distribution (B) in America.

Genotype distribution shows high variability among the five macro-areas studied, ranging between 70.4% (Central Asia) and 15.5% (Southeast Asia) for G1, 39.7% (Pacific area) and 1.9% (Southeast Asia) for G2, 66.7% (Southeast Asia) and 0.4% (Pacific Asia) for G3, 30.8% (Southeast Asia) and 0.5% (Pacific and South Asia) for G6 and 3.7% (Southeast Asia) and 0.1% (Pacific and East Asia) for G4. Only few cases of G5 are reported and all from South and Southeast areas (Table 2).

Asia Pacific, High Income: Data coming from this zone are available only from Japan and South Korea and show a prevalence of HCV of 1.1%, ranging between 1.5% in Japan and 0.8% in the South Korea, with a viraemic rate estimated at 70.5% (Figure 4A).

The most common genotype is G1 (58.7%), followed by G2 (39.7%). Only small percentages of G3, G4 and G6 and no cases of G5 are reported (Table 4). In the only two countries studied G1 and G2 represent over the 90% of the totality of the genotypes observed (Figure 4B).

Central Asia: The countries studied in this area are Armenia, Azerbaijan, Georgia, Kazakhstan, Kyrgyzstan,

Mongolia, Tajikistan, Turkmenistan, Uzbekistan.

The prevalence of HCV in the general population is 5.8% (Table 2), ranging between 11.3% in Uzbekistan and 2.5% in Kyrgyzstan (Figure 4A), with a viraemic rate estimated at 48.7%. No adult HCV prevalence and/or viraemic data are available from Armenia.

The predominant genotype in this area is clearly G1 (70.4%), followed by G3 (19.6%) and G2 (8.6%). Only a small percentage of mixed genotypes were found, whereas no G4, G5 and G6 cases are reported (Table 4).

In Mongolia and Tajikistan, G1 is almost the only genotype found (98.8% and 82.7%, respectively), whereas in two of the five countries in which genotype distribution was studied (Georgia and Uzbekistan) is over the half of the totality of the genotypes identified (62.0% and 67.1%). A considerable percentage of G3 was described in Armenia (37.0%), Georgia (27.0%) and Uzbekistan (26.0%). No genotypes distribution data are available from Azerbaijan, Kazakhstan, Kyrgyzstan and Turkmenistan (Figure 4B).

East Asia: The HCV prevalence in this macroarea, including only China and Taiwan, is 2.8%, ranging between 4.4% in Taiwan and 1.3% in China (Figure

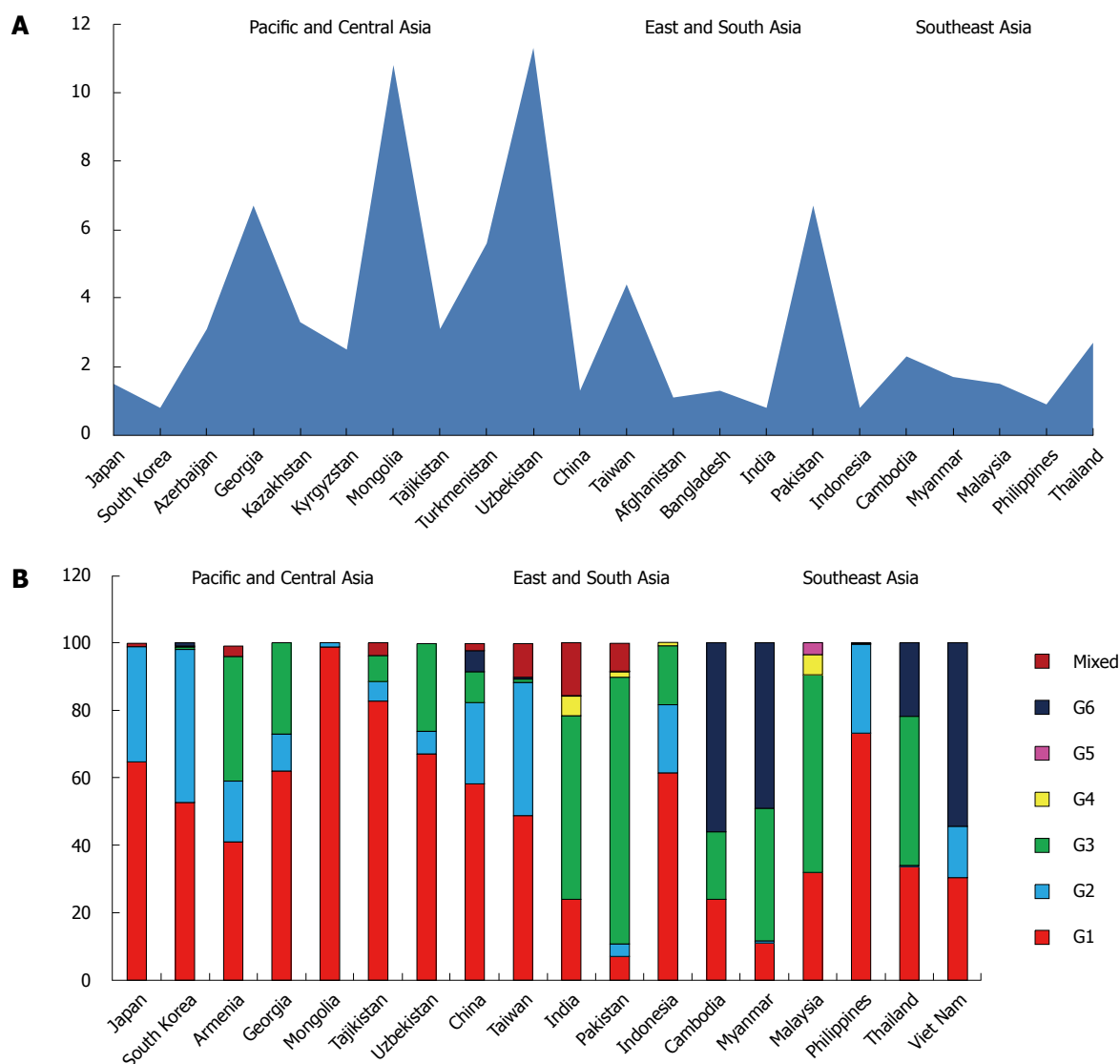


Figure 4 Anti-hepatitis C virus prevalence (A) and genotype distribution (B) in Asia.

4A), with a viraemic rate estimated at 63.6% (Table 2).

The predominant genotypes in this area are G1 (53.5%) and G2 (31.7%). Only a small percentage of G3 and G6 (5.4% and 3.3%, respectively) have been found, whereas no G5 cases are reported (Table 4).

In the only two countries studied, G1 and G2 represent over the 80% of the totality of the genotypes observed, although a significant portion of G3 is described in China (9.1%) (Figure 4B).

South Asia: The prevalence of anti-HCV in the general population of this large area, including Afghanistan, Bangladesh, India and Pakistan, is 2.5%, ranging between 6.7% in Pakistan and 0.8% in India, with a viraemic rate estimated at 78.5% (Figure 4A).

The most common genotype is G3 (66.7%), followed by G1 (15.5%) and G4 (3.7%). Low percentage of G2, G5 and G6 are reported (Table 4). In both the studied countries (India and Pakistan) G3 counts over the half of all the genotypes described (54.4% and 79.0%).

No genotypes distribution data are available from Afghanistan and Bangladesh (Figure 4B).

Southeast Asia: This area includes Cambodia, Indonesia, Laos, Myanmar, Malaysia, Philippines, Sri Lanka, Thailand and Viet Nam, where the prevalence of anti-HCV in the general population is 1.6%, ranging between 2.7% in Thailand and 0.8% in Indonesia (Figure 4A), with a viraemic rate estimated at 60.5%. No adult HCV prevalence and/or viraemic data are available from Laos, Sri Lanka and Viet Nam.

G1 and G6 account over the 60% of all the genotypes identified (35.2% and 30.8%, respectively), followed by G3 (19.9%) and G2 (11.1%). Only a small percentage of G4 and G5 has been found (Table 4). Except for Laos where G6 represents about the totality of genotypes observed (95.6%) and Indonesia and Philippines in which G1 accounts over the 60% of the totality of cases reported, a high heterogeneity is observed in all the other countries. In some countries

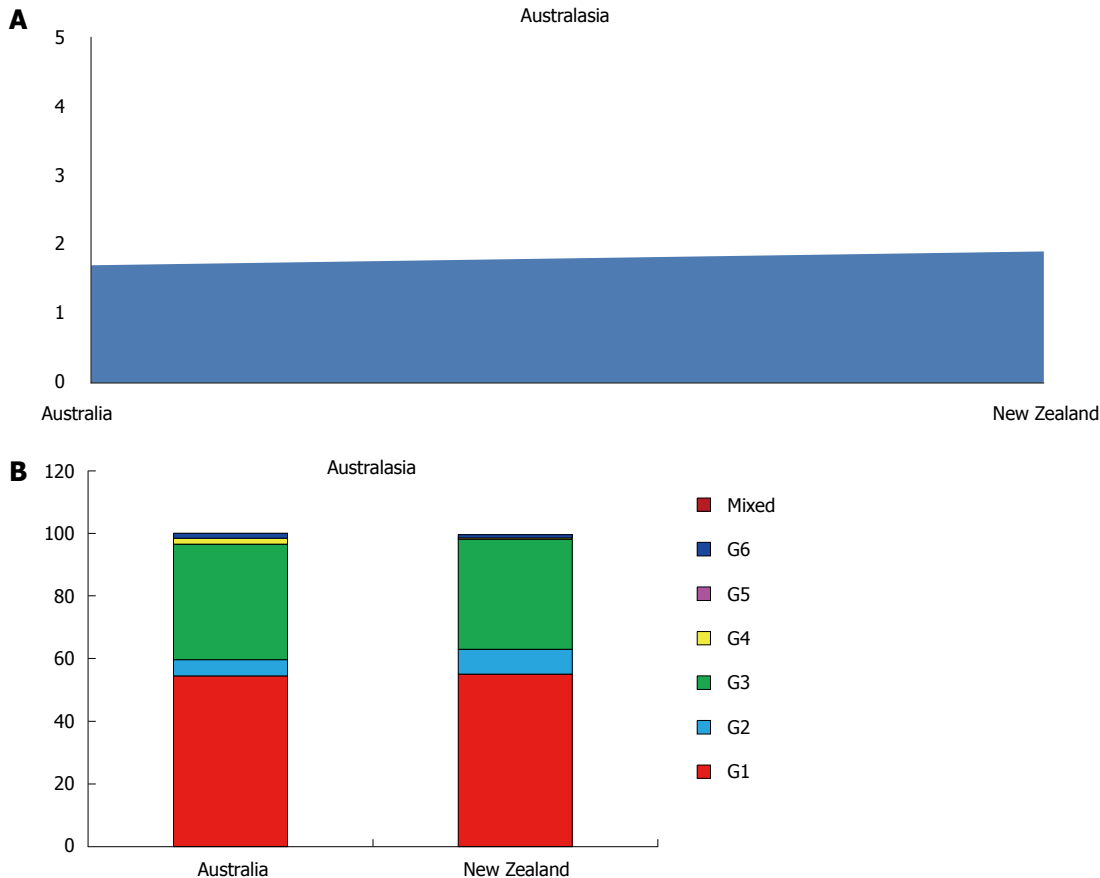


Figure 5 Anti-hepatitis C virus prevalence (A) and genotype distribution (B) in Australasia.

(Cambodia, Myanmar and Viet Nam) G6 is predominant (56.0%, 49.0% and 54.4%, respectively), whereas in others (Sri Lanka, Philippines and Indonesia) G2 shows a significantly percentage (37.5%, 26.4% and 20.2%). Malaysia and Thailand are the countries where G3 shows the highest percentages (58.6% and 44.2%) (Figure 4B).

Australasia

Studies concerning this area has been found only from Australia and New Zealand, with a prevalence of HCV of 1.8%, ranging between 1.9% in New Zealand and 1.7% in Australia, accounting 0.5 millions of estimated cases (Figure 5A). The viraemic rate is 74.8%, with 0.4 million of active HCV replication cases (Table 1).

The more common genotype is G1 (55.0%) followed by G3 (36.0%) and G2 (6.6%). Only a small percentage of G4 and G5 has been found. No G5 cases are described (Table 3, Figure 5B).

Europe

The GBD subdivides European countries into 3 main areas: Central, Eastern and Western.

The estimated prevalence of HCV of the whole continent is 1.8%, accounting over 13 million of estimated cases. The average HCV viraemic rate is 72.4%, with a population of almost 10 million of HCV RNA positive patients (Table 1).

The predominant genotype is G1 (64.4%), followed by G3 (25.5%), G2 (5.5%) and G4 (3.7%). Only small percentages of G5, G6 and mixed or not further classified genotypes are reported (Table 3).

Genotype distribution does not show high variability among the three macro-areas studied, ranging between 70.0% (Central Europe), 68.1% (Eastern Europe) and 55.1% (Western Europe) for genotype 1, 29.0% (Western Europe), 26.6% (Eastern Europe) and 21.0% (Central Europe) for genotype 3.

G2 seems to have a major prevalence in the Western Europe (8.9%), if compared to Eastern (4.3%) or Central (3.2%), whereas G4 is present especially in Central and Western area (4.9% and 5.8%, respectively).

Only few cases of G5 and G6 are reported and mainly from Western area (Table 4).

Central Europe: This large area includes countries like Albania, Bulgaria, Bosnia and Herzegovina, Czech Republic, Croatia, Hungary, Macedonia, Montenegro, Poland, Romania, Serbia, Slovakia and Slovenia, with a prevalence of HCV infection of 1.3%, varying between 1.4% in Slovakia and 0.7% in Czech Republic (Figure 6A) and a viraemic rate estimated at 76.6% (Table 2). We have not found representative data concerning the HCV prevalence and/or HCV viraemic rate from published studies in Albania, Bosnia and Herzegovina, Croatia, Macedonia, Montenegro, Serbia and Slovenia.

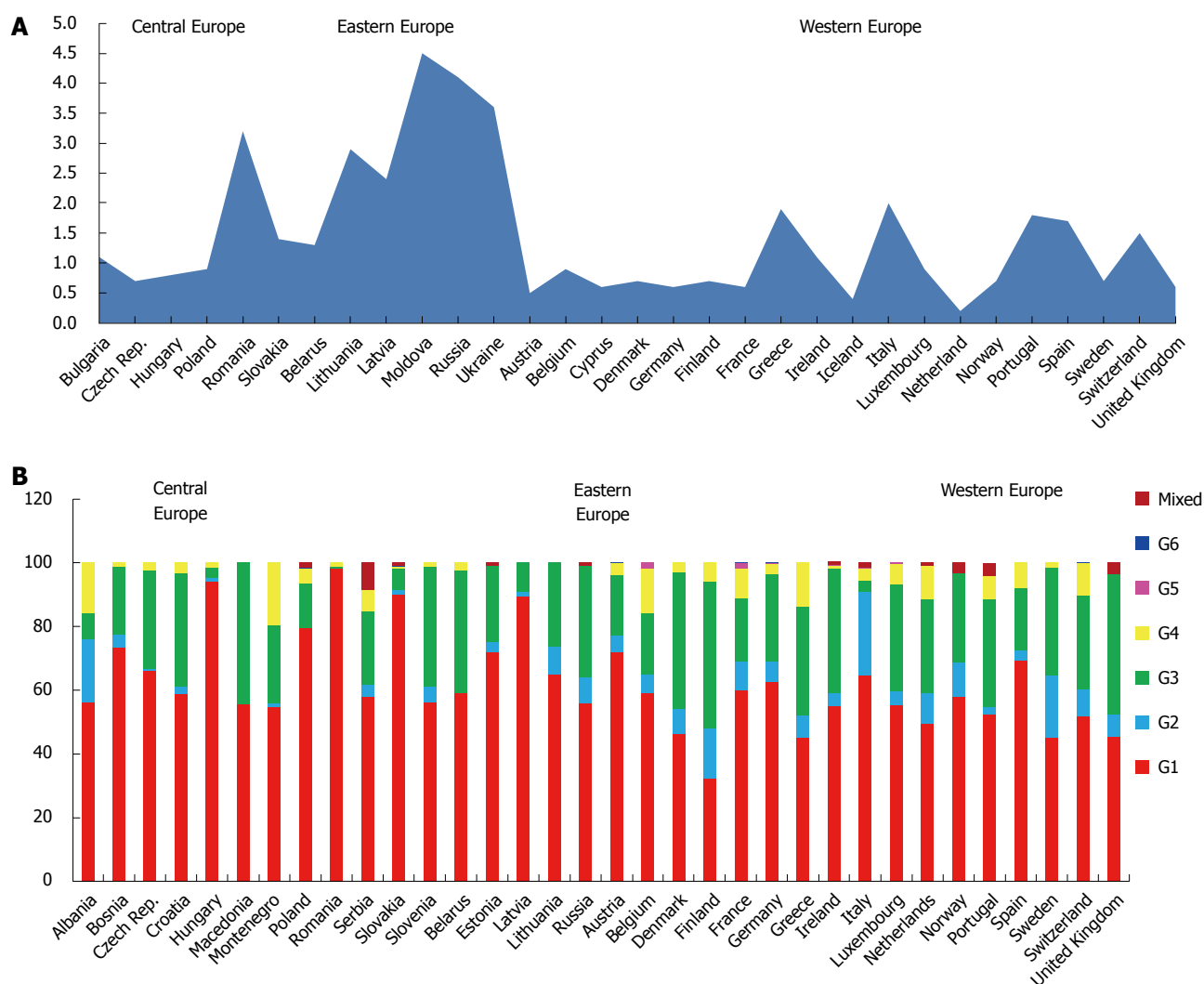


Figure 6 Anti-hepatitis C virus prevalence (A) and genotype distribution (B) in Europe.

The predominant genotypes in this area is G1 (70.0%), followed by G3 (21.0%), G4 (4.9%) and G2 (3.2%). Only a small percentage of mixed genotypes and G6 has been found, whereas no G5 cases are reported (Table 4). In Romania, Hungary and Slovakia, G1 is almost the only genotype found (98.0%, 94.1% and 89.9%, respectively). A considerable percentage of G3 was described in Macedonia (44.6%), Slovenia (37.8%) and Croatia (35.6%), while a significant prevalence of G2 was described only in Albania (20.0%) and of G4 in Montenegro (19.6%) and Albania (16.0%). No genotypes distribution data are available from Bulgaria (Figure 6B).

Eastern Europe: The prevalence of HCV infection in this zone, including Belarus, Estonia, Lithuania, Latvia, Moldova, Russia and Ukraine, is 3.1%, ranging between 4.5% in Moldova and 1.3% in Belarus (Figure 6A), with a viraemic rate estimated at 69.6% (Table 2). No adult HCV prevalence and/or viraemic data are available from Estonia.

The predominant genotypes in this area is G1

(68.1%), followed by G3 (26.6%) and G2 (4.3%). Only a small percentage of mixed genotypes and G4 (0.5%) are reported, whereas no G5 and G6 cases has been described (Table 4).

Only in Latvia G1 is the dominant genetic variant (89.2%). A considerable percentage of G3 was described in Belarus (38.5%) and Russia (35.1%). No genotypes distribution data are available from Moldova and Ukraine (Figure 6B).

Western Europe: The countries studied in this area are Austria, Belgium, Cyprus, Denmark, Finland, France, Germany, Greece, Iceland, Ireland, Italy, Luxembourg, Netherlands, Norway, Portugal, Spain, Sweden, Switzerland and United Kingdom

The prevalence of HCV in the general population of this area is 0.9%, ranging between 2.0% in Italy and 0.2% in Netherlands (Figure 6A), with a viraemic rate estimated at 71.0% (Table 2).

The predominant genotypes is G1 (55.1%), followed by G3 (29.0%), G2 (8.9%) and G4 (5.8%), whereas only small percentages of G5, G6 and mixed

genotypes are reported (Table 4). In Austria, Spain, Germany and Italy G1 is over the sixty percent of all the genotypes found. A considerable percentage of G3 was described in some of the countries of the Northern Europe, as Finland (46.0%), United Kingdom (43.8%), Denmark (43.0%), whereas only Italy shows a significant percentage of G2 (26.0%) (Figure 6B). No genotypes distribution data are available from Cyprus and Iceland.

DISCUSSION

Hepatitis C virus (HCV) infection is one of the main global health burden^[5,39-43] and its paradigm varies regionally depending on its historical and present risk factors. A country-specific policy of prevention, diagnosis and treatment could reduce this disease burden, but unfortunately, in many countries, there is a lack of robust epidemiological data upon which to base these strategies, being HCV infection rates^[2,44-48] typically focused on quantifying only the anti-HCV prevalence with no attention to HCV genotypes distribution.

This study represents one of the most comprehensive effort to present in a systematic manner the actual situation of the epidemiology of HCV infection, using the best available published and unpublished data between 2000 and 2015, giving a special care not only on available data, but focusing the attention especially on more relevant data. For example, studies on HCV prevalence among blood donors, available in many countries and surely attractive for the large sample size, were excluded because this population, corresponding to healthy screened adults, is not representative of the total population. For the same reason, even if opposite, numerous high risk populations studies (*e.g.*, PWIDs, haemodialysis patients, cancer patients, *etc.*) were not considered too. Finally, it is important to clear that all the studies published prior to 2000 were also excluded considering the global epidemiological changes that HCV infections has had in the latest twenty years^[39,40,49-52].

Studying 138 countries worldwide (36 in Sub-Saharan Africa, 16 in Americas, 26 in Asia, 2 in Australasia, 19 in North Africa/Middle East area and 39 in Europe), total global HCV prevalence is estimated at 2.5% (177 million of HCV infected adults). Central Asia and Central Africa are estimated to have high prevalence (> 3.5%); East, South and Southeast Asia, West and East Africa, North Africa and Middle East, Southern and Tropical Latin America, Caribbean, Australasia, and Eastern Europe moderate prevalence (1.5%-3.5%); whereas Southern Africa, North America, Andean and Central Latin America, Pacific Asia and Western and Central Europe have low prevalence (< 1.5%) (Table 2).

No adult HCV prevalence studies were available from 19 countries (4 in Asia, 4 in Americas, 5 in North Africa/Middle East area and 6 in Europe). In order of their contribution, twenty five of the 138 countries

studied account for almost the half of total viraemic infections worldwide and China, Egypt, India, Nigeria, Pakistan and Russia together for more than 70% of them.

Our analysis shows that globally the prevalence and number of HCV infected patients, if compared to a similar study concerning the period 1990-2005^[2], has decreased from 2.8% [95% uncertainty interval (UI): 2.6%-3.1%] to 2.5% (95%UI: 2.3%-2.7%) and from 185 to 177 millions. It is interesting to note that the most relevant decrease has been observed in the high income zones, especially in Western Europe (-1.5%), Southern Africa (-1.2%) and Australasia (-0.9%), whereas a massive increase it's reported in some of the low income areas as Central Africa (+3.7%) and Central Asia (+2.0%)^[53].

By estimating the total number of HCV RNA positive infections, our data show that the global average viraemic rate is at 67% (118.9 million of HCV RNA positive cases), varying from 48.7% in Central Asia to 80.2% in Tropical Latin America (Table 2). It is interesting notice that some countries, as Poland, where it has been reported a high anti-HCV prevalence and an increasing viraemic rate^[54], using a confirmatory antibody test, the anti-HCV prevalence is significantly lower (< 1%)^[55], suggesting that some historically high antibody prevalence estimates may be influenced by the use of low sensitive screening HCV tests.

Globally, G1 accounts for 49.1% of all anti-HCV infections among adults making it the most common, followed by G3 (17.9%), G4 (16.8%), G2 (11.0%), G5 (2.0%) and G6 (1.4%). Our data are quite different from those reported by other global studies^[56], not only because we considered some countries before excluded, but mostly because our data were summed with other global or continental reports^[43,57,58]. Undefined or mixed genotypes accounts for 1.8% of the total HCV infections (Table 1). However, significant regional, country and local variations exists. Infections in Caribbean, Latin America, North America and Europe are predominately caused by G1 (83.0%, 74.3%, 66.3% and 64.4%, respectively). North Africa and the Middle East has a large G4 population (65.3%), probably attributable to the high prevalence of G4 in Egypt (93.1%). If Egypt is excluded, in fact, G4 accounts only for 32.3% of all infections and the genotype distribution of this region is dominated by G1 (48.3%). In Asia the two most common genotypes are G1 (46.6%) and G3 (22.4%), the latter largely driven by its high prevalence in South Asia (66.7%), where in India and Pakistan G3 shows the highest percentages of the whole continent (54.4% and 79.0%, respectively). In Australasia, G1 dominated (55.0%), followed by G3 (25.5%). HCV genotypes by sub regions are shown in Table 4.

Aside from countries entirely lacking genotype information (Bulgaria, Cyprus, Moldova and Ukraine in Europe, Afghanistan, Azerbaijan, Bangladesh,

Kazakhstan, Kyrgyzstan and Turkmenistan in Asia, Bolivia, Martinique, Suriname and Uruguay in America, Israel, Oman, Qatar and Yemen in North Africa/Middle East Area and several of the Western and Eastern Sub-Saharan African countries) our data suggest that data concerning the genotype prevalence are particularly weakest in the majority of Asia (accounting for 3.6% of the global population), followed by Africa (3.2% of the global population) and Latin America (1.4% of the global population), if considered in terms of proportion of the genotyped patients respect to overall populations.

It is interesting to note that G1, the most prevalent genotype in high income countries, well served by second generation DAA therapies with a viral eradication rates > 90%^[59,60], is also the most prevalent globally. This may be related to the strict association between G1 and the transfusion risk very common prior to the discovery of HCV in 1989^[22].

The high circulation of G3, instead, the second most common genotype, especially in Europe, which accounts for almost 20% of global infections, not susceptible to the first generation of DAA protease inhibitors and less susceptible to Sofosbuvir^[61,62], is likely related to the association between subtype 3a and drug abuse^[20,63,64] and also to migrations to Europe from countries where this subtype is dominant, such as India and Pakistan^[65].

Although HCV G1 and G3 infections are the most prevalent globally (67.0% if considered together), other genotypes are particularly common in lower-income countries (Table 4), as G2 in West Africa (62.9%) and in some regions of South America, probably caused by population movements during the trans-Atlantic slave trade in the Eighteenth Century^[26] and G4 and G6, highly present in Central and Northern Africa (82.9% and 65.3%) and in Southeast Asia (30.8%). The particularly high prevalence of subtype 4a in Egypt could be the result of unsafe injections during the anti-schistosomal public health campaigns in the past^[66], whereas the diffusion of subtype 4d in Europe^[67,68] and of subtype 6a in Hong Kong^[69] have probably been amplified by the high number of PWIDs in these areas.

It has been estimated that G2, G4, and G6 combined account for nearly one third of all HCV cases globally. Higher proportions of G2 were found in Northern Europe and in some of the ex-soviet republics, probably in accordance to the Asian genotypes distribution, and in Italy, especially in the Southern regions^[70,71].

Anyway, it is necessary to clarify that this analysis shows several limitations, especially related to the lack of information available from some extended regions (first of all, Africa and Asia) that has necessarily forced us to use regional estimates, sometimes coming from few high populated countries. For example, the Central Asia viraemic rate estimate, calculated on the basis of data published in Uzbekistan (viraemic rate of 39%) and in Mongolia (viraemic rate of 70%), is heavily

influenced by Uzbekistan due to its much larger population. Data from additional countries would be helpful in the future in minimizing this bias.

Another limitation is the lack of robust epidemiology studies at the national level. Only 21% of the 138 countries included in this analysis shows a sample size > 10000, selected by multiple cities or regions, and a random sampling strategy, while the majority are generally conducted in a select population within one setting (e.g., hospital, clinic, city, etc.).

Anyway, in the absence of better data, this analysis suggests that HCV prevalence and the viraemic rate have decreased from 2005 to date, maybe for the impact of the new DAA therapies, even if a better knowledge of genotype distribution at a national level, especially in its subtype diversification, may be yet critic for the complete understanding of HCV disease and to design and testing of vaccines, especially in countries where genotype diversity is particularly high.

COMMENTS

Background

Hepatitis C virus (HCV) infection and distribution of its genetic variants throughout the world.

Research frontiers

This manuscript provided the strategies to fight the diffusion of HCV infection worldwide.

Innovations and breakthroughs

The authors showed us the epidemiological up-date of HCV genotypes.

Applications

These findings can be used in clinical therapy and prevention strategies.

Peer-review

This is a pretty good manuscript. The analysis performed by the authors maximized the extraction of meaningful information from available literature.

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Double-layered covered stent for the treatment of malignant oesophageal obstructions: Systematic review and meta-analysis

Zeiad Hussain, Athanasios Diamantopoulos, Miltiadis Krokidis, Konstantinos Katsanos

Zeiad Hussain, Athanasios Diamantopoulos, Konstantinos Katsanos, Department of Interventional Radiology, Guy's and St. Thomas' Hospitals, NHS Foundation Trust, London SE1 7EH, United Kingdom

Miltiadis Krokidis, Department of Interventional Radiology, Addenbrooke's Hospital, Cambridge University Hospitals NHS Foundation Trust, Cambridge CB2 0QQ, United Kingdom

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Correspondence to: Konstantinos Katsanos, MSc, MD, PhD, EBIR, Consultant Vascular and Interventional Radiology, Department of Interventional Radiology, Guy's and St. Thomas' Hospitals, NHS Foundation Trust, King's Health Partners, London SE1 7EH, United Kingdom. konstantinos.katsanos@gstt.nhs.uk
Telephone: +44-207-1885550
Fax: +44-207-9288071

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Abstract

AIM

To investigate the efficacy of double-layered covered stent in the treatment of malignant oesophageal obstructions.

METHODS

A systematic review and meta-analysis was performed following the PRISMA process. PubMed (Medline), EMBASE (Excerpta Medical Database), AMED (Allied and Complementary medicine Database), Scopus and online content, were searched for studies reporting on the NiTi-S polyurethane-covered double oesophageal stent for the treatment of malignant dysphagia. Weighted pooled outcomes were synthesized with a random effects model to account for clinical heterogeneity. All studies reporting the outcome of palliative management of dysphagia due to histologically confirmed malignant oesophageal obstruction using double-layered covered nitinol stent were included. The level of statistical significance was set at $\alpha = 0.05$.

RESULTS

Six clinical studies comprising 250 patients in total were identified. Pooled technical success of stent insertion was 97.2% (95%CI: 94.8%-98.9%; $I^2 = 5.8\%$). Pooled complication rate was 27.6% (95%CI: 20.7%-35.2%; $I^2 = 41.9\%$). Weighted improvement of dysphagia on a scale of 0-5 scoring system was -2.00 [95%CI: -2.29%-(-1.72%); $I^2 = 87\%$]. Distal stent migration was documented in 10 out of the 250 cases examined.

Pooled stent migration rate was 4.7% (95%CI: 2.5%-7.7%; $I^2 = 0\%$). Finally, tumour overgrowth was reported in 34 out of the 250 cases with pooled rate of tumour overgrowth of 11.2% (95%CI: 3.7%-22.1%; $I^2 = 82.2\%$). No funnel plot asymmetry to suggest publication bias (bias = 0.39, $P = 0.78$). In the sensitivity analysis all results were largely similar between the fixed and random effects models.

CONCLUSION

The double-layered nitinol stent provides immediate relief of malignant dysphagia with low rates of stent migration and tumour overgrowth

Key words: Double-layered covered stent; Malignant oesophageal obstructions; Dysphagia; Double-layered nitinol stent

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Core tip: The aim of this systematic review and meta-analysis is to investigate the efficacy of double-layered nitinol covered stent in the treatment of malignant oesophageal obstructions. The literature was searched following the PRISMA selection process as recommended by Cochrane. Weighted pooled outcomes were synthesized with a random effects model to account for clinical heterogeneity. This meta-analysis highlights the promising outcomes of the double-layered covered stent and demonstrates significant immediate relief of dysphagia with low rates of stent migration and tumour overgrowth. The unique design of this stent seems to combine the merits of both plain covered and uncovered metal esophageal stent designs.

Hussain Z, Diamantopoulos A, Krokidis M, Katsanos K. Double-layered covered stent for the treatment of malignant oesophageal obstructions: Systematic review and meta-analysis. *World J Gastroenterol* 2016; 22(34): 7841-7850 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i34/7841.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i34.7841>

INTRODUCTION

Oesophageal cancer is the sixth leading cause of cancer-related deaths worldwide. Despite the significant advances in diagnostic and therapeutic options, the 5-year survival rate remains remarkably low^[1]. More than 50% of patients present late and are deemed unsuitable for surgical resection^[2,3]. The lack of early warning signs and symptoms means that patients usually become symptomatic in an advanced stage of the disease. In addition, esophageal cancer usually affects elderly patients who suffer from multiple co-morbidities. Both these factors render affected patients poor surgical candidates^[1,2,4]. The

most important complaint of these patients is the tumour related dysphagia resulting in poor oral intake and malnutrition.

Palliative treatment options of malignant dysphagia include balloon dilatation, laser ablation, sclerotherapy, thermal coagulation, radiotherapy, chemotherapy and endoscopically and/or fluoroscopically guided stent placement across the obstruction. Nowadays, the placement of self-expanding metal stents (SEMS) is considered the treatment of choice for malignant dysphagia, given the quick and effective symptomatic relief and its ease of use and relative safety^[2,3,5]. Stents used in for the treatment of malignant esophageal obstruction can be either covered or uncovered^[6-9].

One of the more widely used SEMS today is the double-layered covered nitinol stent (NiTi-S, Taewoong Medical, South Korea) which was designed to combine the advantageous characteristics of covered stents that prevent tissue ingrowth and re-obstruction, and uncovered stents that have demonstrated lower rates of migration. The authors performed a systematic review of the literature in order to assess the clinical outcomes of this double-layered covered nitinol stent in the treatment of malignant esophageal obstruction with a focus on stent insertion success, peri-procedural complications, relief of dysphagia, and stent failure because of migration or tumour overgrowth.

MATERIALS AND METHODS

Search strategy

The literature was searched following the PRISMA selection process as recommended by Cochrane. PubMed (Medline, 1950 to present), EMBASE (Excerpta Medical Database, 1980 to present), AMED (Allied and Complementary Database, 1985 to present), Scopus (1970 to present) and the CENTRAL (Cochrane Central Register of Controlled Trials) were searched. The search terms used were "oesophageal" or "stent" or "double-covered" or "NiTi-S" and "trial" or "study" or "controlled trial", as well as the relevant terms and corresponding Medical Subjects Headings (MeSH). The search was last updated on 31 January 2016. There were no language restrictions. Papers of all relevant published studies identified from the above search strategy were obtained and assessed for potential eligibility independently by two of the senior authors (AD and KK).

Selection criteria

All studies reporting the outcome of palliative management of dysphagia due to histologically confirmed malignant oesophageal stricture/obstruction in adult patients, by insertion of a double-layered covered nitinol stent were included in the present meta-analysis. There was no intention of future resection or surgery in all included patients due to locally advanced disease, distant metastasis or serious co-morbidities

precluding surgical management. Stent placement was performed either under fluoroscopy or endoscopic guidance.

Papers reporting the management of oesophageal strictures due to benign disease, anastomotic strictures and stents inserted as a bridge to surgery or prior to radical radiotherapy were excluded from this study. Studies reporting the outcomes of stents other than the double-layered covered NiTi-S stent were also excluded.

Data extraction

Data were extracted by the same two authors independently. Descriptive data including baseline demographics, follow-up periods and primary and secondary outcome measures were collected. Outcome measures were defined according to the previously published International guidelines. These included immediate technical success of stent insertion, peri-procedure complication rates, reported dysphagia improvement, and rates of stent migration and tumour overgrowth during follow-up. The same dysphagia scoring system was used in all papers as follows: score 0, normal diet; score 1, able to eat some solid food; score 2, able to eat some semi-solids only; score 3, liquids only; and score 4, complete inability to swallow^[10]. Data were extracted from the main text and tables of the published papers. In case of missing data, relevant abstracts and presentations from annual meetings proceedings were analysed and/or the corresponding authors were contacted. Any disagreement was resolved by consensus between the investigators.

Stent design

The double-layered covered nitinol stent (NiTi-S, Taewoong Medical, Seoul, South Korea) has a double-layer configuration, consisting of an inner "dog-bone" nitinol stent (nickel-titanium thermal memory alloy) that is fully covered with polyurethane layer and an outer uncovered nitinol wire mesh designed to prevent or reduce stent migration by allowing tumour in-growth and anchorage at the tumour site^[11]. When delivered, the stent flares at its proximal and distal ends. A thread is attached inside the proximal flange of the stent, when being pulled; the thread reduces the diameter of the stent, enabling stent repositioning or removal. The Niti-S stent shortens approximately 35% after placement, therefore, a stent measuring 2 to 4 cm longer than the stricture is usually used to allow for a 1- to 2-cm extension above and below the proximal and distal components of the tumour. The stent is delivered in a compressed form inside an introducer sheath under fluoroscopic or endoscopic guidance.

Statistical analysis

Quantitative data synthesis of all included studies was created/performed using Statsdirect software (Version

2.7.9, Statsdirect Ltd, Cheshire, United Kingdom). Categorical variables were expressed in percentages and continuous variables in mean \pm SD if normally distributed. Summary statistics of the primary and secondary endpoint measures were expressed as weighted proportional outcomes and the associated 95% CIs. The random DerSimonian and Laird (D-L) effects model was applied to calculate the pooled proportional outcomes. We also performed a sensitivity analysis with the inverse variance fixed effects model. Cochran's Q (χ^2) test and I^2 statistic were used to test for statistical evidence of heterogeneity (non-combinability of studies) across the included studies. An I^2 value of less than 25% indicates low heterogeneity, while values between 25% and 50% and values of more than 50% indicate moderate and high heterogeneity, respectively. Potential publication bias was assessed by visual inspection of inverted funnel plot asymmetry as recommended for systematic reviews and meta-analyses including a small number of studies. The Horbold-Egger test was also used to indicate publication bias in case of subjective funnel plot evaluation. The level of statistical significance was set at $\alpha = 0.05$.

RESULTS

Systematic review

A total of 6 articles meeting the inclusions criteria of this study were identified with the PRISMA selection process (Figure 1) including 5 single-arm cohort studies and 1 randomized controlled trial^[11-16]. Clinical details of a total of 250 patients (206 male), median age is 68 (range: 42-84), who underwent palliative stent insertion for malignant oesophageal obstruction over a period of 10 years (January 2006-January 2016) were obtained and pooled for the purposes of this analysis. The median follow-up period ranged between 2 mo (62 d) and 6 mo^[11-16]. Two studies reported comparisons between different stents (single and double-layered stents)^[12,13], but we extracted and pooled only the double-layered ones for the purposes of the meta-analysis. Histology of treated tumours was balanced between squamous cell carcinoma and adenocarcinoma and nearly half of the cases involved the distal oesophagus. A summary of characteristics of the studies included in this meta-analysis is shown in Table 1.

Meta-analysis

Predefined outcome measures were reported in all 6 included studies. Successful stent insertion was reported in 244 out of the 250 cases. The pooled technical success rate (weighted proportion) was 97.2% (95%CI: 94.8%-98.9%; Figure 2). There was low statistical heterogeneity ($I^2 = 5.8\%$, $P = 0.38$) and no visual asymmetry of the respective funnel plot to suggest publication bias (bias = -1.48, $P = 0.08$).

Complications were reported in 70 out of the 250

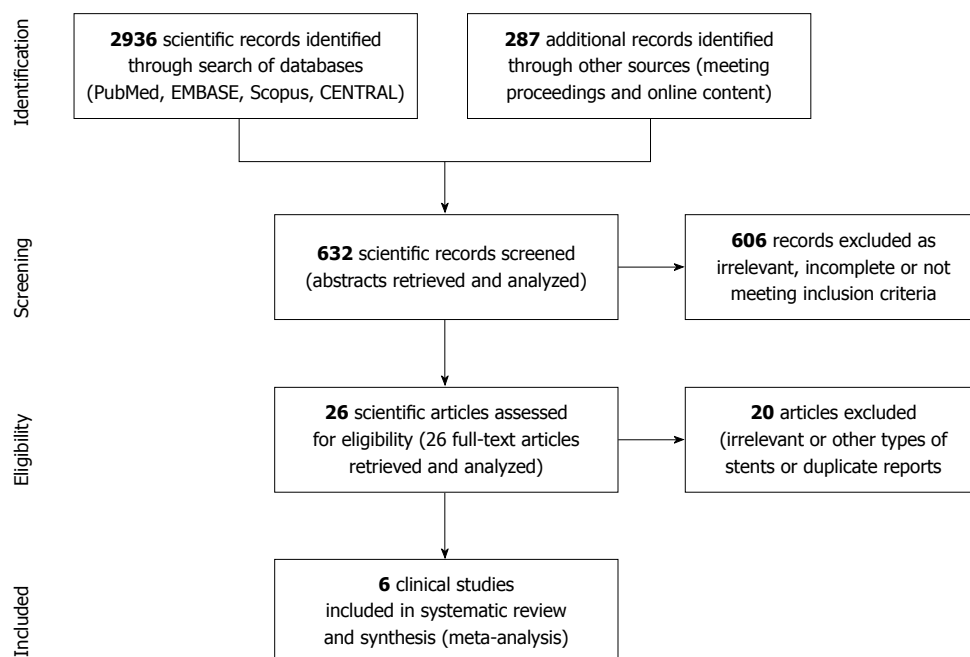


Figure 1 PRISMA selection. Study selection process according to the PRISMA statement by the Cochrane Collaboration.

Table 1 Design and patient characteristics of included clinical trials

Publication	Study design	Sample (n)	Age (yr)	Male gender	Adenocarcinoma	Distal esophagus	Tumour length (cm)
Verschuur <i>et al</i> ^[16] , 2006	Prospective cohort	42	65 ± 14	60%	76%	21%	7.8 ± 2.4
Kim <i>et al</i> ^[13] , 2009	Prospective randomized	17	68 ± 8	94%	33%	39%	6.1 ± 2.6
Park <i>et al</i> ^[13] , 2010	Prospective cohort	32	67 ± 9	75%	N/A	53%	N/A
Battersby <i>et al</i> ^[12] , 2012	Prospective cohort	55	72	67%	56%	93%	11.7 ± 2.5 (stent length)
Kim <i>et al</i> ^[14] , 2012	Prospective cohort	48	68 ± 11	81%	33%	37%	N/A
Mezes <i>et al</i> ^[11] , 2014	Retrospective cohort	56	N/A	61%	38%	55%	N/A

N/A: Not available.

cases. Most often encountered complications were reflux esophagitis and aspiration pneumonia, whereas oesophageal fistula was rarely noted (Table 2). Pooled complication rate was 27.6% (95%CI: 20.7%-35.2%; Figure 3). There was moderate statistical heterogeneity ($I^2 = 41.9\%$, $P = 0.13$) and no funnel plot asymmetry to suggest publication bias (bias = -1.21, $P = 0.79$).

Pooled improvement in dysphagia score (weighted score reduction compared to baseline) was -2.00 [95%CI: -2.29-(-1.72); Figure 4]. There was high statistical heterogeneity ($I^2 = 87\%$, $P < 0.0001$) and no evidence of publication bias (bias = -3.79, $P = 0.46$).

Distal stent migration was documented in 10 out of the 250 cases examined. Pooled stent migration rate was 4.7% (95%CI: 2.5%-7.7%; Figure 5). There was very low statistical heterogeneity ($I^2 = 0.0\%$, $P = 0.82$) and no funnel plot asymmetry to suggest publication bias (bias = 0.39, $P = 0.78$).

Finally, tumour overgrowth was reported in 34 out of the 250 cases in total. Pooled overgrowth rate was 11.2% (95%CI: 3.7%-22.1%; Figure 6). There was high statistical heterogeneity ($I^2 = 82.2\%$, $P < 0.0001$) and some funnel plot asymmetry suggestive of potential publication bias (bias = 4.13, $P = 0.06$).

In the sensitivity analysis all results were largely similar between the fixed and random effects models as summarised in Table 3.

DISCUSSION

The use of SEMS is a well-established palliative management of the dysphagia associated with advanced oesophageal malignancy, but the optimal stent design is still debated^[2,7,17]. Stents used in the treatment of oesophageal obstruction are made of stainless steel, nitinol or plastic stents and they can be either covered

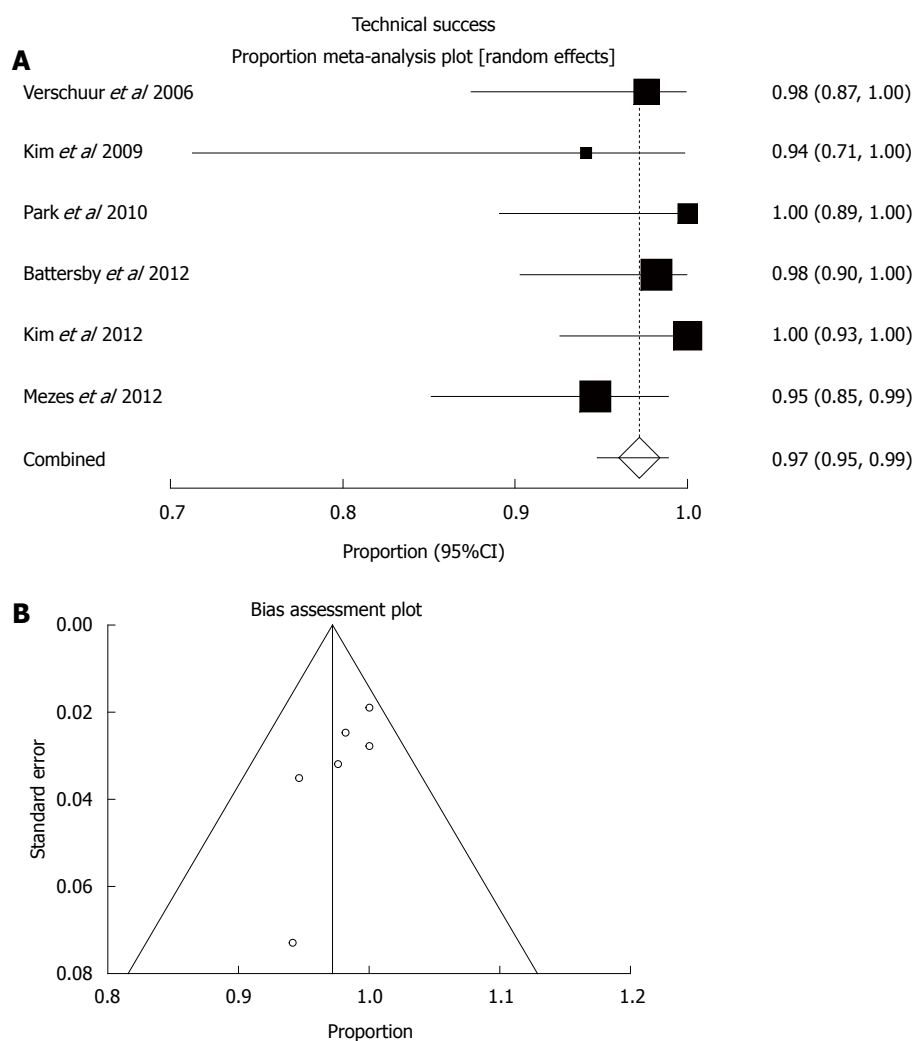


Figure 2 Technical success. A: Random effects forest plot of weighted pooled estimate; B: Respective funnel plot for bias assessment (the standard error of the proportion was plotted against the proportion for each study).

Table 2 Event counts of tumour overgrowth, stent migration and complications encountered

Publication	Tumour overgrowth	Stent migration	Food impaction	Reflux esophagitis	Aspiration pneumonia	Esophageal fistula	Perforation	Hemorrhage
Verschuur <i>et al</i> ^[16] , 2006	<i>n</i> = 2	<i>n</i> = 3	-	<i>n</i> = 2	<i>n</i> = 2	-	<i>n</i> = 1	<i>n</i> = 1
Kim <i>et al</i> ^[13] , 2009	-	-	<i>n</i> = 1	-	-	<i>n</i> = 1	-	-
Park <i>et al</i> ^[13] , 2010	<i>n</i> = 5	<i>n</i> = 1	-	<i>n</i> = 4	<i>n</i> = 1	<i>n</i> = 1	-	-
Battersby <i>et al</i> ^[12] , 2012	<i>n</i> = 1	<i>n</i> = 3	6%	8%	3%	0.8%	0.4%	-
Kim <i>et al</i> ^[14] , 2012	<i>n</i> = 13	<i>n</i> = 1	<i>n</i> = 2	<i>n</i> = 13	<i>n</i> = 5	<i>n</i> = 2	-	-
Mezes <i>et al</i> ^[11] , 2014	<i>n</i> = 19	<i>n</i> = 4	-	-	-	<i>n</i> = 1	-	-

or uncovered^[2-5]. Previous covered plastic stents have now been largely replaced with metal stent which provide safe, rapid and effective symptomatic relief with fewer complications. Covering of metal struts with polyethylene, polytetrafluoroethylene (PTFE), silicone or polyurethane coating is believed to reduce the rate of re-obstruction due to tissue ingrowth/overgrowth compared to the uncovered ones^[6-9], however, they are implicated in higher rates of migration^[2]. Newer commercially available stent options include biodegradable oesophageal stents and intraluminal radioactive

stents^[18,19].

Newer stents are constructed from nitinol, which has the advantage of thermal memory and can conform to a more predictable self-expanding shape after deployment compared to older metal stents made from stainless steel^[2,8,19]. The new type of double-layered nitinol stent is believed to combine the merits of both covered and uncovered stents. Its inner layer is covered with polyurethane material to prevent tumour encroachment, while the outer layer consists of an uncovered metal mesh to prevent or reduce

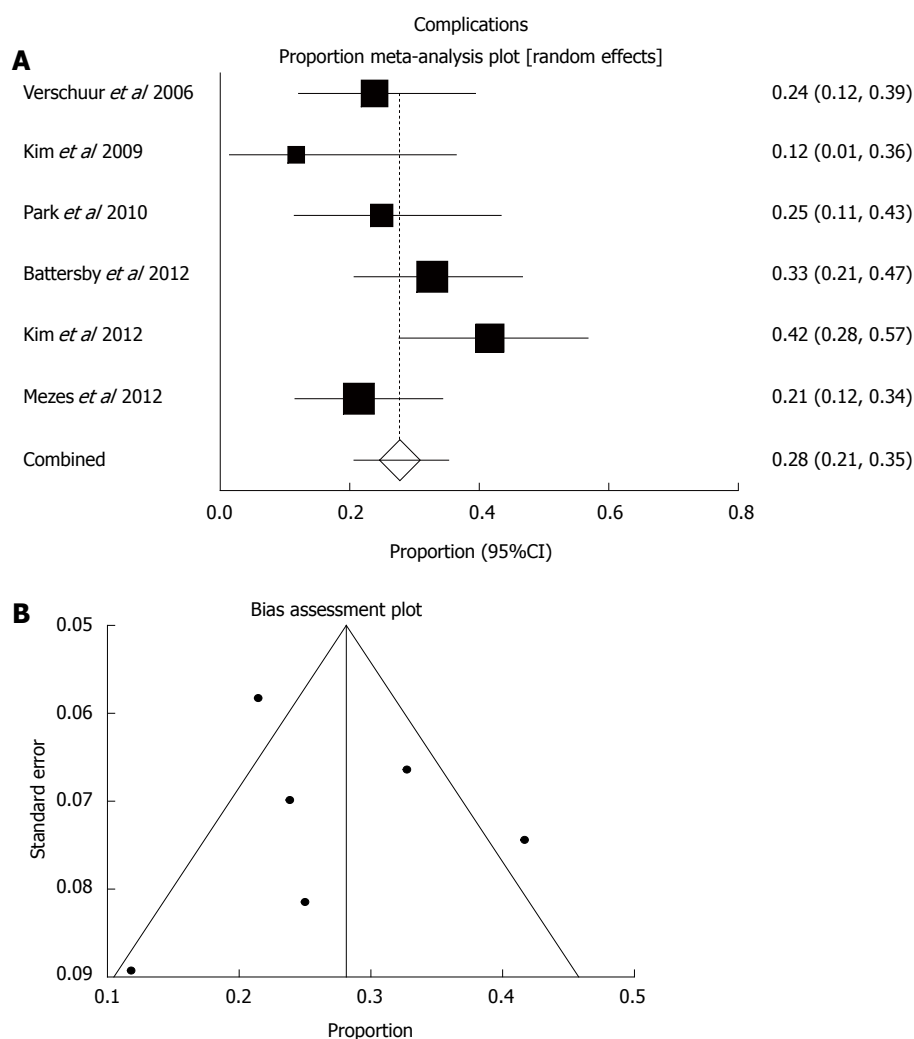


Figure 3 Complications. A: Random effects forest plot of weighted pooled estimate; B: Respective funnel plot for bias assessment (the standard error of the proportion was plotted against the proportion for each study).

migration by being implanted into tumour tissue or oesophageal wall^[11-16]. The present meta-analysis has demonstrated a high pooled success of stent insertion (> 97%) with acceptable rates of procedure-related complications. The latter were encountered in a quarter of the cases (27.6%) and seem to be in line with the usually encountered complications during oesophageal stent insertion for palliation of cancer-related dysphagia^[2,4,12]. Most importantly, significant relief of dysphagia was found that was 2 points on average out of the 0-4 dysphagia scale. This is very important to help restore oral intake and treat poor nutrition that has been found to be associated with poorer long-term survival outcomes^[11]. Furthermore, the double-layered covered Niti-S stent was found to be related to quite low incidence of stent migration and late tumour overgrowth compared to historical outcomes of uncovered or single-covered stents^[2,3].

The most frequent early and late complication of oesophageal SEMS is stent malfunction or failure, either due to migration (*i.e.*, slipping distally into the stomach or small bowel), or tissue ingrowth/

overgrowth or less frequently food impaction that leads to recurrent dysphagia. The pooled estimate of migration rate in this analysis was 4.7%, which on one hand is significantly lower than the so far reported migration rates of covered stents that range between 25%-32%, and on the other hand appears to be very similar to the reported migration rates of uncovered stents^[2]. Stent migration will present with early recurrent dysphagia and may be easily treated with endoscopic retrieval of the migrated stent followed by repeat insertion of another one, or may develop to more serious small bowel obstruction mandating open laparotomy that entails a risk of death in the setting of advanced oesophageal cancer^[12]. Symptomatic tumour overgrowth at the stent edge was found to affect nearly 1 in 10 of the analysed cases and can be easily treated with repeat co-axial insertion of another covered stent. Stent failure/blockage because of food impaction was rarely reported and is a more benign complication that may be treated with endoscopic clearance of the clogged debris and encouragement of fizzy drinks^[2,3].

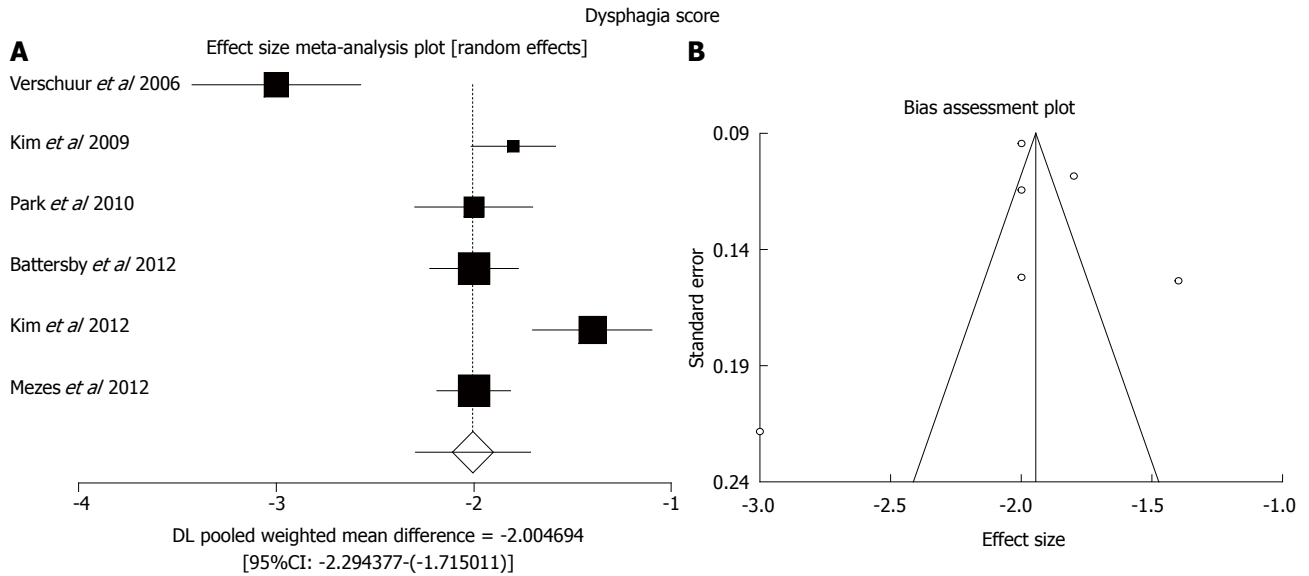


Figure 4 Improvement of dysphagia score. A: Random effects forest plot of weighted pooled treatment effect; B: Respective funnel plot for bias assessment (the standard error of the score was plotted against the effect size for each study).

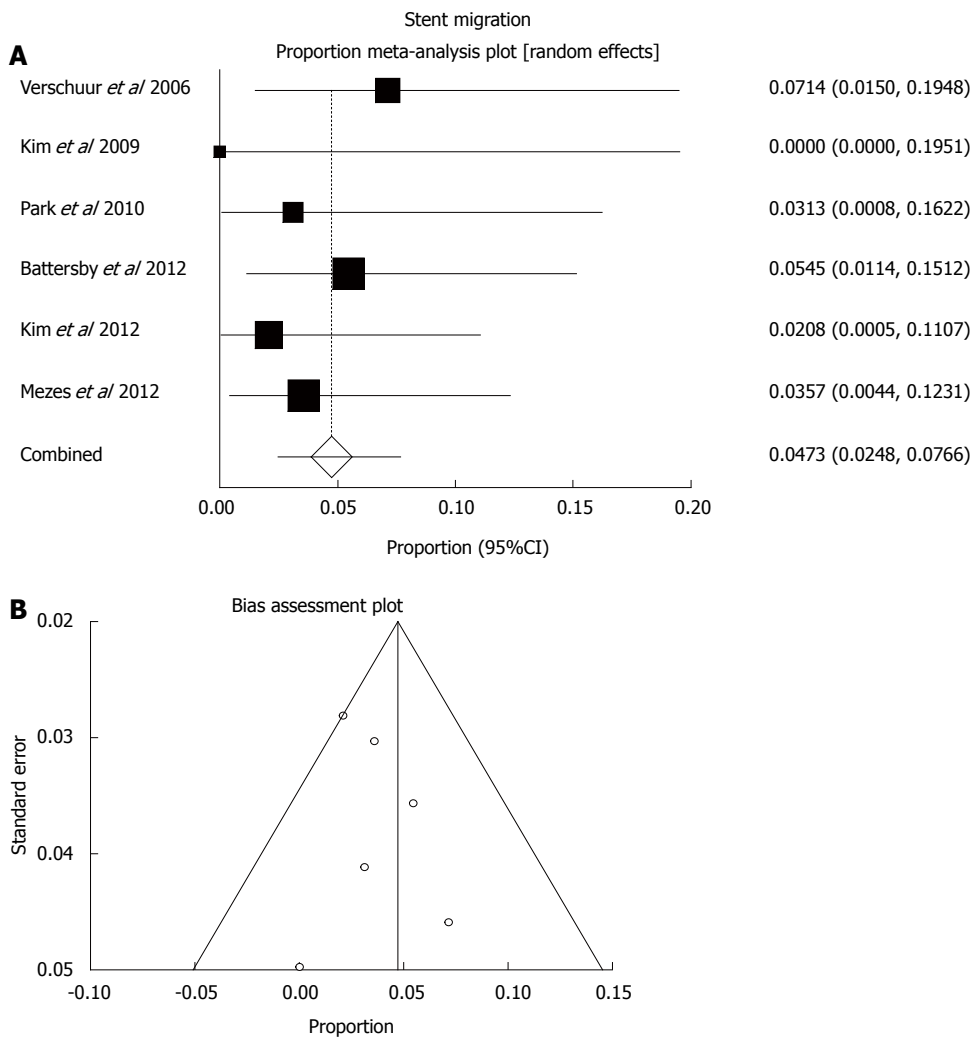


Figure 5 Stent migration. A: Random effects forest plot of weighted pooled estimate; B: Respective funnel plot for bias assessment (the standard error of the proportion was plotted against the proportion for each study).

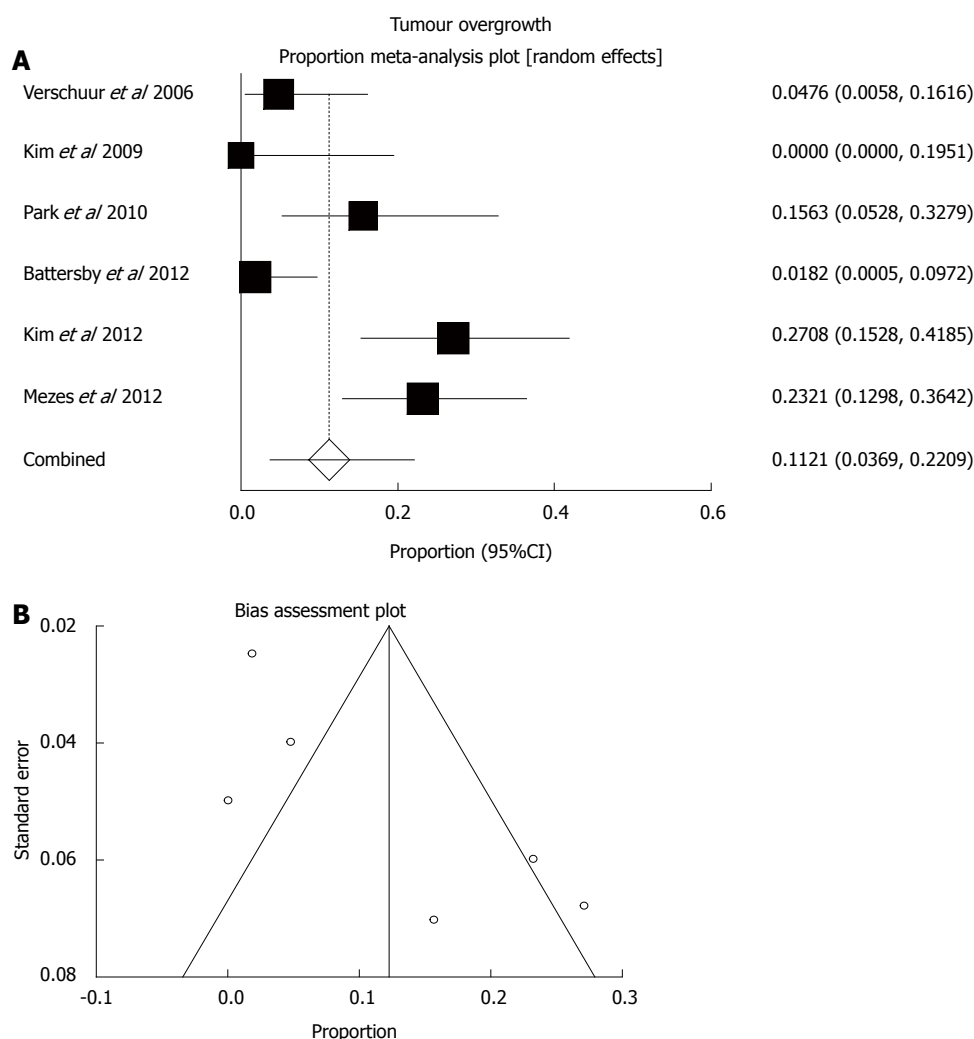


Figure 6 Tumour overgrowth. A: Random effects forest plot of weighted pooled estimate; B: Respective funnel plot for bias assessment (the standard error of the proportion was plotted against the proportion for each study).

Table 3 Summary of the meta-analysis for all outcome measures with the random and fixed effects models

Parameters	Pooled estimates Random (95%CI)	Pooled estimates Fixed (95%CI)	I ² heterogeneity
Technical success (%)	97.2 (94.8-98.9)	97.2 (94.9-98.9)	Low (5.8%)
Overall complications (%)	27.6 (20.7-35.2)	28.1 (22.8-33.8)	Moderate (41.9%)
Dysphagia score improvement (0-4)	-2.00 [-2.29-(-1.72)]	-1.94 [-2.04-(-1.84)]	High (87.0%)
Migration (%)	4.7 (2.5-7.7)	4.7 (2.5-7.7)	Low (0.0%)
Overgrowth (%)	11.2 (3.7-22.1)	12.3 (8.5-16.6)	High (82.2%)

Other frequently reported complications of the double-layered PTFE-covered Nitinol stent include reflux esophagitis, most commonly associated with lower oesophageal and gastro-oesophageal junction (GOJ) tumours and the infrequent but life threatening aspiration pneumonia and tracheo-oesophageal fistula. Hence, prescription of proton-pump inhibitors (PPI) is recommended in all cases of covered stent placement across the GOJ. The proposed mechanism of fistula formation after stent placement is pressure necrosis of the tumour and oesophageal wall or trauma from the sharp stent ends on the oesophageal mucosa^[12].

This complication has been treated successfully with a second overlapping covered stent into the first stent^[20]. Other rarely reported complications may include haemorrhage and oesophageal perforation because of poor operative technique and/or local tumour infiltration.

Study limitations

The main limitation of this meta-analysis is the small number of studies identified, and subsequently, the relatively small number of total patients included in the analysis. There was some between-trials heterogeneity

that may compromise internal validity, but there was good agreement between the random and fixed effects models that underlines the external validity of our results. In addition, with the exception of 1 randomized design, no comparative studies were available to allow comparison of different stent designs. The number of trials and sample size were too small to support further subgroup comparisons or other regression analyses.

The data compiled in this meta-analysis highlight the promising outcomes of the double-layered polyurethane covered NiTi-S stent in the treatment of esophageal obstruction secondary to malignancy with significant immediate relief of dysphagia and low rates of stent migration and tumour overgrowth. Despite the inherent limitations of this study, the present data suggests that patients are likely to benefit from the unique design of this stent which seems to have low migration rate, comparable to that of uncovered stent, while maintaining the lower rate of re-obstruction due to tissue ingrowth/overgrowth seen in plain covered stents^[12]. The authors of this study therefore believe that the double-layered covered nitinol stent combines the merits of both plain covered and uncovered metal esophageal stent designs.

COMMENTS

Background

Esophageal cancer is the sixth leading cause of cancer-related deaths worldwide. More than 50% of patients present late and are deemed unsuitable for surgical resection. The most important complaint of these patients is the tumour related dysphagia resulting in poor oral intake and malnutrition. Nowadays, the placement of self-expanding metal stents is considered the treatment of choice for malignant dysphasia given the quick and effective symptomatic relief and its ease of use and relative safety.

Research frontiers

Stents used in the treatment of oesophageal obstruction are made of stainless steel, nitinol or plastic stents and they can be either covered or uncovered. Previous covered plastic stents have now been largely replaced with metal stents which provide safe, rapid and effective symptomatic relief with fewer complications. Covering of metal struts is believed to reduce the rate of re-obstruction compared to the uncovered ones, however, they are implicated in higher rates of migration. Newer stents are constructed from nitinol, which has the advantage of thermal memory and can conform to a more predicable self-expanding shape after deployment. The new type of double-layered nitinol stent is believed to combine the merits of both covered and uncovered stents.

Innovations and breakthroughs

This is the first systematic review of the literature in order to assess the clinical outcomes of this double-layered covered nitinol stent in the treatment of malignant esophageal obstruction with a focus on stent insertion success, peri-procedural complications, relief of dysphagia, and stent failure because of migration or tumour overgrowth.

Applications

The data compiled in this meta-analysis highlight the promising outcomes of the double-layered polyurethane covered NiTi-S stent in the treatment of esophageal obstruction secondary to malignancy with significant immediate relief of dysphagia and low rates of stent migration and tumour overgrowth.

Peer-review

This study showed that patients are likely to benefit more from the unique

design of this double-layered covered nitinol stent. It is very nicely presented and convincingly shows the very satisfactory outcome following the use of the double covered stent.

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Immunohistochemical evaluation for outflow reconstruction using opened round ligament in living donor right posterior sector graft liver transplantation: A case report

Yukihiro Sanada, Yasunaru Sakuma, Hideki Sasanuma, Atsushi Miki, Takumi Katano, Yuta Hirata, Noriki Okada, Naoya Yamada, Yoshiyuki Ihara, Taizen Urahashi, Naohiro Sata, Yoshikazu Yasuda, Koichi Mizuta

Yukihiro Sanada, Takumi Katano, Yuta Hirata, Noriki Okada, Naoya Yamada, Yoshiyuki Ihara, Taizen Urahashi, Koichi Mizuta, Department of Transplant Surgery, Jichi Medical University, Shimotsuke City, Tochigi 329-0498, Japan

Yasunaru Sakuma, Hideki Sasanuma, Atsushi Miki, Naohiro Sata, Yoshikazu Yasuda, Department of Surgery, Jichi Medical University, Shimotsuke City, Tochigi 329-0498, Japan

Author contributions: Sanada Y contributed to study design, acquisition of data, analysis and interpretation of data, and drafting of the manuscript; Sasanuma H, Miki A, Katano T, Hirata Y, Okada N, Yamada N, Ihara Y and Urahashi T contributed to acquisition of data, and analysis and interpretation of data; Sakuma Y, Sata N, Yasuda Y and Mizuta K contributed to analysis and interpretation of data, and critical revision of the manuscript for important intellectual content; all authors read and approved the final manuscript.

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Correspondence to: Yukihiro Sanada, MD, PhD, Department of Transplant Surgery, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke City, Tochigi 329-0498, Japan. yuki371@jichi.ac.jp
Telephone: +81-285-587069
Fax: +81-285-587069

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Abstract

Utilizing the opened round ligament as venous grafts during liver transplantation is useful but controversial, and there are no pathological analyses of this procedure. Herein, we describe the first reported case of a pathological analysis of an opened round ligament used as a venous patch graft in a living donor liver transplantation (LDLT). A 13-year-old female patient with biliary atresia underwent LDLT using a posterior segment graft from her mother. The graft had two hepatic veins (HVs), which included the right HV (RHV; 15 mm) and the inferior RHV (IRHV; 20 mm). The graft RHV and IRHV were formed into a single orifice using the donor's opened round ligament (60 mm × 20 mm) as a patch graft during bench surgery; it was then anastomosed end-to-side with the recipient inferior vena cava. The recipient had no post-transplant complications involving the HVs, but she died of septic shock with persistent cholangitis and jaundice 86 d after LDLT. The HV anastomotic site had no stenosis or thrombus on autopsy. On pathology, there was adequate patency and continuity between the reci-

patient's HV and the donor's opened round ligament. In addition, the stains for CD31 and CD34 on the inner membrane of the opened round ligament were positive. Hepatic venous reconstruction using the opened round ligament as a venous patch graft is effective in LDLT, as observed on pathology.

Key words: Opened round ligament; Venous patch graft; Hepatic venous reconstruction; Living donor liver transplantation; All-in-one venoplasty

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Core tip: Utilizing the opened round ligament as venous grafts during liver transplantation is useful but controversial, and there are no pathological analyses of this procedure. Herein, we describe the first reported case of pathological analysis of an opened round ligament used as a venous patch graft in living donor liver transplantation. The hepatic venous (HV) anastomotic site had no stenosis or thrombus on autopsy. On pathology, there was adequate patency and continuity between the recipient's HV and the donor's opened round ligament. In addition, the stains for CD31 and CD34 on the inner membrane of the opened round ligament were positive.

Sanada Y, Sakuma Y, Sasanuma H, Miki A, Katano T, Hirata Y, Okada N, Yamada N, Ihara Y, Urahashi T, Sata N, Yasuda Y, Mizuta K. Immunohistochemical evaluation for outflow reconstruction using opened round ligament in living donor right posterior sector graft liver transplantation: A case report. *World J Gastroenterol* 2016; 22(34): 7851-7856 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i34/7851.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i34.7851>

INTRODUCTION

The use of the opened round ligament as a venous patch graft has become accepted in hepatopancreatobiliary surgery because of its easy availability^[1-3]. Recent studies have demonstrated the use of the opened round ligament as a venous patch graft in hepatic venous reconstruction during living donor liver transplantation (LDLT)^[4-7]. However, there is no consensus regarding the efficacy of using the opened round ligament as a venous patch graft. Although there are reports on the radiological patency of the opened round ligament after LDLT and reports on the pathological assessment of its epithelium at the time of transplant^[8,9], pathological analyses of long-term patency and continuity after LDLT have not been reported.

Herein, we describe the first reported case of a pathological analysis on an autopsy specimen of an opened round ligament used as a venous patch graft in hepatic venous reconstruction during LDLT. Approval

to conduct this study was obtained from the Ethics Committees of Jichi Medical University.

CASE REPORT

A 13-year-old female patient with biliary atresia was considered for LDLT because of decompensated liver cirrhosis with jaundice. Her body height and weight were 159.4 cm and 54.0 kg, respectively, and her standard liver volume was 1095 mL. The blood test results were as follows: white blood cells 9600/ μ L; hemoglobin 8.7 g/dL; hematocrit 26.0%; platelets 149000/ μ L; albumin 2.9 g/dL; creatinine 0.35 mg/dL; total bilirubin 31.6 mg/dL; aspartate aminotransferase 93 mU/mL; alanine aminotransferase 50 mU/mL; prothrombin time-international normalized ratio 1.40; and activated partial thromboplastin 58.3 s. The model for end-stage liver disease score was 23.

The patient underwent an ABO-compatible LDLT using her mother's posterior segment graft (390 g; graft volume to standard liver volume ratio: 35.6%). The graft had two hepatic veins (HVs), including the right hepatic vein (RHV; 15 mm) and the inferior RHV (IRHV; 20 mm) (Figure 1A). The graft RHV and IRHV were formed into a single orifice using the donor's opened round ligament (60 mm \times 20 mm) as a venous patch graft during bench surgery (Figure 1B); it was then anastomosed end-to-side with the recipient inferior vena cava (Figure 1C and D). Portal vein reconstruction using an interposition vein graft from the right saphenous vein of the donor was performed between the recipient portal vein and the graft posterior portal vein. Hepatic artery reconstruction using microsurgical technique was performed between the recipient right hepatic artery and the graft posterior hepatic artery. For biliary reconstruction, a Roux-en-Y hepaticojejunostomy was performed. Intraoperative color Doppler ultrasonography was performed to assess the blood flow velocity and pattern after vascular reconstruction, and the flow velocity and pattern were satisfactory. The length of the operation was 21 h and 8 min, and the bleeding volume was 7438 mL. Tacrolimus and methylprednisolone were used for standard post-operative immunosuppressive therapy.

On post-operative day (POD) 8, portal vein thrombosis was detected by color Doppler ultrasonography, and the patient was given intravenous urokinase. On POD 26, acute cellular rejection was diagnosed by a graft liver biopsy, and she was treated with pulse steroids. On POD 40, cytomegalovirus viremia was detected by the C7-HRP test, and she underwent preemptive anti-cytomegalovirus therapy. On POD 48, portal vein balloon dilatation was performed by interventional radiology after the progressive narrowing of the portal vein anastomotic stricture to less than 2 mm was detected by color Doppler ultrasonography. During percutaneous transhepatic portography of the intrahepatic portal vein, bilhemia was detected, and

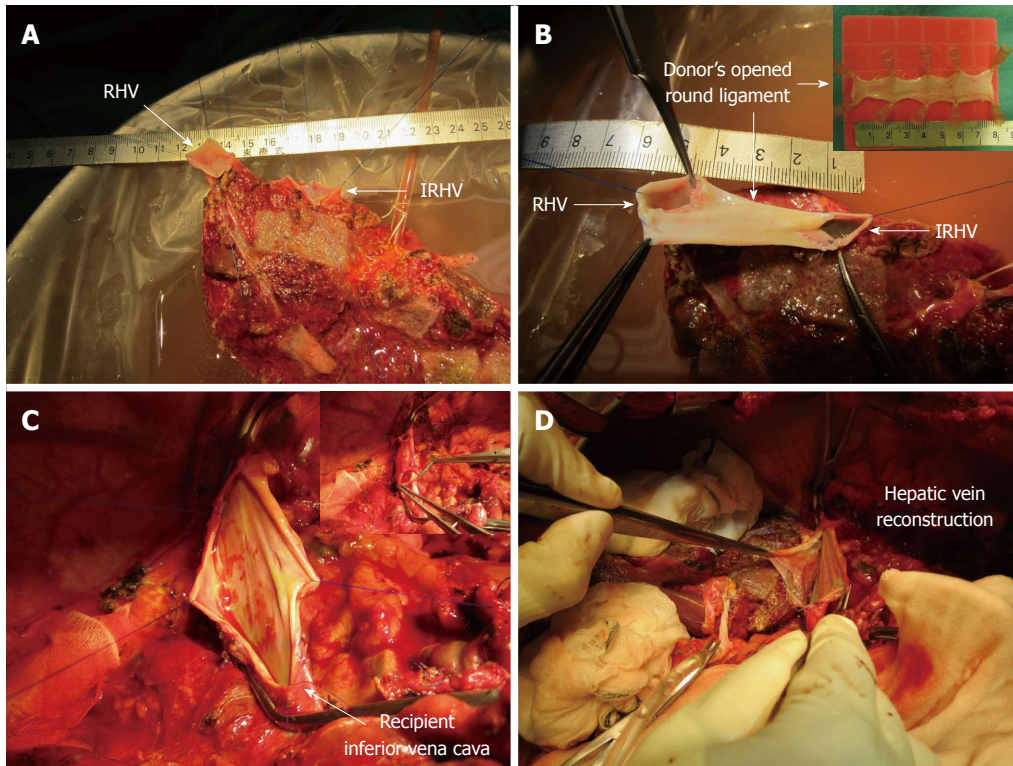


Figure 1 The graft had two hepatic veins. Including the right hepatic vein (RHV; 15 mm) and the inferior RHV (IRHV; 20 mm) (A). The graft RHV and IRHV were formed into a single orifice using the donor's opened round ligament (60 mm × 20 mm) as a venous patch graft during bench surgery (B). It was then anastomosed end-to-side with the recipient inferior vena cava (C and D).

thereafter, she suffered from persistent jaundice (total bilirubin 6.4 mg/dL). On POD 86, she developed a fever with liver dysfunction, and antibiotic treatment for acute cholangitis was administered. However, she died of septic shock with persistent cholangitis and bacteremia (with *Serratia marcescens*) on POD 88.

There were no post-transplant complications involving the HVs, and the radiological patency between the opened round ligament and the HV was confirmed on POD 82 (Figure 2). The HV anastomotic site had no stenosis or thrombus on an autopsy (Figure 3A and B). The patency and continuity between the donor's opened round ligament and the HV were adequate on pathological examination. In addition, the stains for CD31 and CD34 on the inner membrane of the opened round ligament were positive, a finding also observed in the graft HV (Figure 3C and D).

DISCUSSION

Outflow block in LDLT can potentially result in liver congestion, graft failure, and death^[10]. When a right liver graft is used for LDLT and the graft HV is anastomosed end-to-end with the recipient HV or end-to-side with the recipient inferior vena cava, the liver graft expands in all directions in the limited right subphrenic space during liver graft regeneration. Consequently, the HV anastomosis can be compressed and twisted in some circumstances^[11]. In addition,

multiple HV reconstructions are sometimes required in a right liver graft LDLT. Therefore, reports have indicated that it is important to simplify HV reconstruction and enlarge the graft HV orifice by an all-in-one sleeve patch graft venoplasty, which has shown excellent outcomes^[11-14].

The efficacy of various venous grafts used in HV reconstruction has been reported. These grafts include auto-venous grafts (from the inferior mesenteric vein, gonadal vein, external iliac vein, internal jugular vein, and saphenous vein)^[13,15,16], venous grafts from a living donor, native portal veins^[17], opened round ligaments^[4-9,12], cryopreserved homografts^[18-22], and polytetrafluoroethylene grafts^[23,24]. However, the harvest of a venous graft from a living donor or the recipient himself is not easy. In addition, the availability of cryopreserved homografts is limited, and the adverse effects of polytetrafluoroethylene grafts are unclear. Alternatively, the opened round ligament is easier to use, and less invasive than other venous grafts, but it is controversial in regard to its long-term patency. Recently, use of the opened round ligament as a venous patch graft in hepatic venous reconstruction during LDLT has been reported^[4-7]. Although there are reports on the radiological patency of the opened round ligament after LDLT and reports on the pathological assessment of its epithelium at the time of transplant^[8,9], there are no pathological analyses of patency and continuity. In this case, we

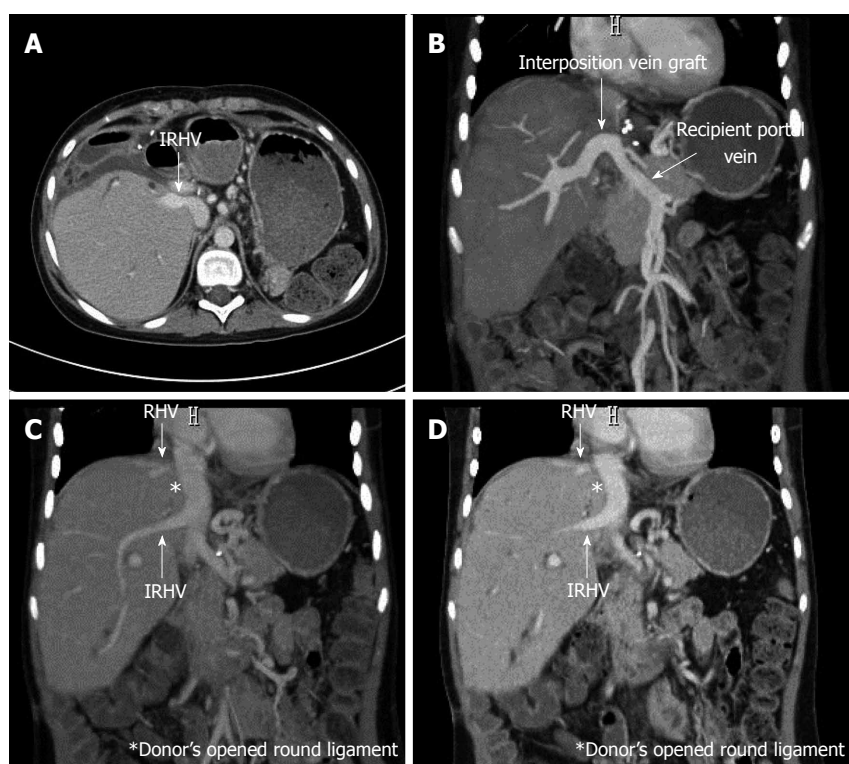


Figure 2 Abdominal enhanced computed tomography on post-operative day 82. The radiological patency of the donor's opened round ligament and the hepatic vein was confirmed (A, B, C, D). RHV: Right hepatic vein; IRHV: Inferior right hepatic vein.

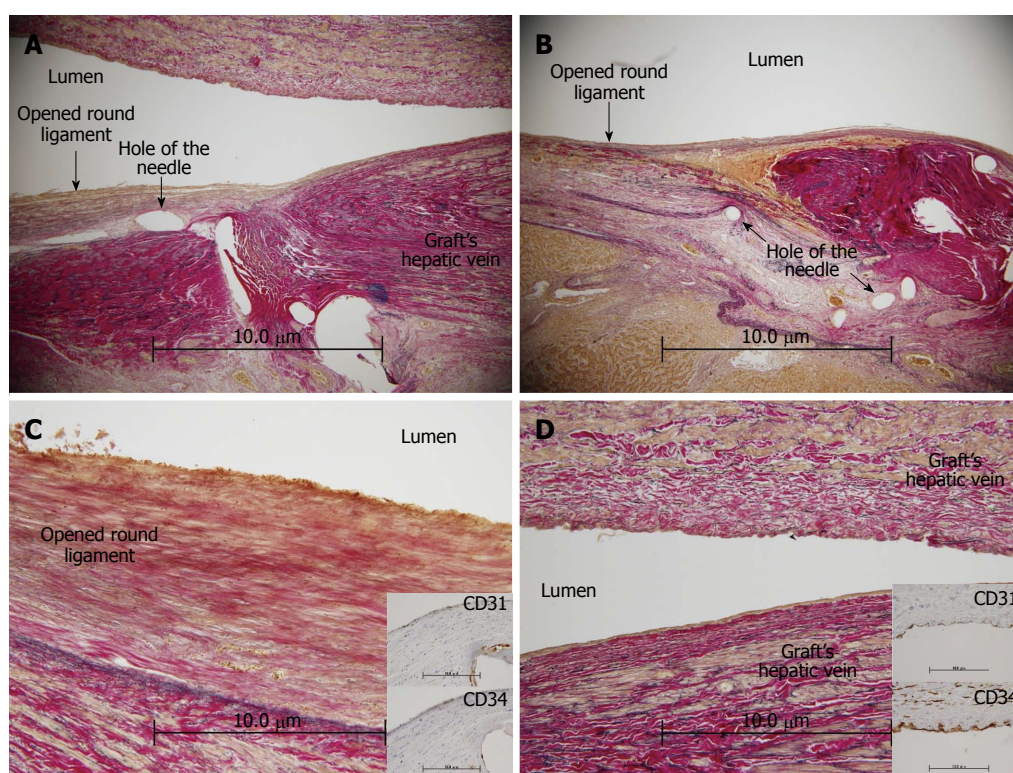


Figure 3 Pathology of the hepatic venous anastomotic site on autopsy. No stenosis or thrombus was identified (A and B). On pathology, there was adequate patency and continuity between the recipient's hepatic vein and the donor's opened round ligament. In addition, the stains for CD31 and CD34 on the inner membrane of the opened round ligament were positive, a finding also observed in the graft hepatic vein (C and D).

conducted a pathological autopsy evaluation of a donor's opened round ligament used as a venous patch graft in LDLT. This is the first report that demonstrates the pathological efficacy of the opened round ligament after surgery. The HV anastomotic site had no stenosis or thrombus (Figure 3A and B). On pathology, there was adequate patency and continuity between the recipient HV and the donor's opened round ligament. In addition, the stains for CD31 and CD34 on the inner membrane of the opened round ligament were positive, a finding also observed in the graft HV (Figure 3C and D). Therefore, we believe that the pathological patency and the existence of venous endothelial cells support the efficacy of using the opened round ligament as a venous patch graft.

In conclusion, using the opened round ligament as a venous patch graft is easy, less invasive, and had been shown to be pathologically effective. The accumulation of further cases and the long-term observation of this case are needed to confirm our findings.

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We thank Professor Akira Tanaka (Department of Pathology, Jichi Medical University) for helpful advices regarding pathological evaluations.

COMMENTS

Case characteristic

A 13-year-old female patient with biliary atresia underwent living donor liver transplantation using a posterior segment graft from her mother, and she died of septic shock with persistent cholangitis and jaundice 86 d after living donor liver transplantation.

Clinical diagnosis

The patient was diagnosed septic shock with persistent cholangitis and jaundice 86 d after living donor liver transplantation.

Differential diagnosis

No-obstructive afferent loop syndrome.

Laboratory diagnosis

Biliary stasis.

Imaging diagnosis

The radiological patency between the opened round ligament and the hepatic vein was confirmed on POD 82.

Pathological diagnosis

On pathology, there was adequate patency and continuity between the recipient's hepatic vein and the donor's opened round ligament. In addition, the stains for CD31 and CD34 on the inner membrane of the opened round ligament were positive, a finding also observed in the graft hepatic vein.

Treatment

The antibiotic treatment for acute cholangitis was administered.

Related reports

The efficacy of various venous grafts used in hepatic vein reconstruction

has been reported. These grafts include auto-venous grafts (from the inferior mesenteric vein, gonadal vein, external iliac vein, internal jugular vein, and saphenous vein), venous grafts from a living donor, native portal veins, opened round ligaments, cryopreserved homografts, and polytetrafluoroethylene grafts.

Term explanation

Opened round ligament and all-in-one venoplasty.

Experiences and lessons

Hepatic venous reconstruction using the opened round ligament as a venous patch graft is effective in living donor liver transplantation, as observed on pathology.

Peer-review

The manuscript provides anecdotal support for the use of opened round ligament in hepatic vein reconstruction.

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