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ABOUT COVER

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OPINION REVIEW

Role of betaine in liver disease-worth revisiting or has the die been cast?

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Abstract

Nonalcoholic steatohepatitis (NASH) is an important indication for liver transplantation in many Western countries due to the epidemic of obesity and insulin resistance. Unfortunately, no medication is approved for NASH and risk factor modification is often advised. Over the last decade, several clinical trials on NASH have been conducted with several ongoing and the future looks promising. Although betaine (trimethyl glycine) was evaluated for NASH, results were mixed in the clinical trials in large part due to the quality of the studies. It seems reasonable to re-evaluate betaine in clinical trials for NASH and alcoholic liver disease due to its low cost, tolerability and mechanism of action.

Key Words: Betaine; Obesity; Insulin resistance; Nonalcoholic steatohepatitis; Cirrhosis; S-adenosyl homocysteine; S-adenosyl methionine

Core Tip: This article revisits the role of betaine in liver disease with a focus on nonalcoholic steatohepatitis. Although a randomized controlled trial did not report benefit over placebo, there were limitations with the study. Given the excellent safety profile and tolerance of betaine, it appears this would be an appropriate time to re-evaluate betaine for liver diseases such as nonalcoholic and alcoholic liver diseases.

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INTRODUCTION

Betaine (or trimethy glycine) is a naturally occurring dietary compound which appears to have a beneficial effect in studies on both alcoholic steatohepatitis (ASH) and nonalcoholic steatohepatitis (NASH). It is also synthesized in the liver by the oxidation of dietary choline and common causes of betaine deficiency are often related to malnutrition from conditions such as chronic alcoholism[1]. Although the mechanisms of its actions are complex, betaine is believed to reverse inflammation, hepatic steatosis and fibrosis by several mechanisms: (1) Acting as a methyl donor for the conversion of homocysteine to methionine during alcohol exposure or in the presence of vitamin B12 and folate deficiency, all of which inhibit methionine synthetase activity; (2) Methylating phosphatidylethanolamine to phosphatidylcholine *via* the Kennedy pathway (an important route in the synthesis of very low density lipoproteins prior to hepatic export); and (3) Restoration of hepatic mitochondrial glutathione and S-adenosyl methionine (SAM): S-adenosyl homocysteine ratios which reduce hepatic oxidative stress and reverse inflammation, hepatic steatosis and fibrosis in experimental and clinical studies[2,3]. As one of betaine's principal actions is to act as a methyl donor, a diet which may be rich in carbohydrates but deficient in choline or betaine will lead to hypomethylation of homocysteine and phosphatidylethanolamine resulting in hepatic steatosis and inflammation. This was demonstrated in an elegant case-control study in which betaine levels were significantly decreased in NASH patients in contrast to patients with nonalcoholic fatty liver disease (NAFLD)[4]. In addition, mutations in mitochondrial dimethyglycine dehydrogenase, an enzyme involved in choline metabolism, were significantly associated with patients who had more advanced liver disease.

Despite these encouraging findings in multiple pre-clinical studies, betaine has not been extensively investigated in large randomized studies for the treatment of nonalcoholic or alcoholic liver disease. Since 2000, only four clinical trials have evaluated betaine for the treatment or NASH but due to limitations with study design, definitive conclusions on the impact of betaine on NASH should not be made (Table 1). For example, the first study by Miglio *et al*[5] did not use histopathology to diagnose NASH and thus no conclusions could be made. The second small case series of ten patients by Abdelmalek *et al*[6] reported biochemical and histological improvement although three patients did not complete the study. This led to a larger, randomized placebo-controlled trial by the same investigator in which 35 patients were randomized to 20 g of anhydrous betaine *vs* placebo for 1 year[7]. The primary aim of the study was to assess the impact of betaine on liver function tests and the secondary aim was to determine its effect on hepatic histology according to the Brunt criteria. The investigators reported betaine had no impact on aminotransferases and in addition, no change in hepatic fibrosis was noted during the study. Fewer betaine-exposed patients *vs* placebo had an improvement in steatosis by more than 1 point (29% *vs* 61%, $P < 0.01$). Although these findings were disappointing, there were several limitations in this study, most salient of which was the high drop-out rate which was attributed to side effects. Interestingly, betaine is now available in both anhydrous form (administered in a solution) or capsules and administration of either a lower dose of betaine anhydrous or capsules may have reduced side effects and improved compliance. This study was also only one year in duration and it would seem unlikely that reversing or improving histology in a disease with an insidious onset and which takes years to develop would occur during such a short space of time. This is now reflected by the current state of clinical trials in NASH which are now several years in duration. The most recent study of betaine in NASH was a case series of 35 patients with histologically confirmed NASH of whom the majority of patients showed stability of disease progression or improvement in hepatic histology after one year of treatment[8]. However, this study was limited by the absence of a control arm.

A prospective, unblinded randomized study of betaine is currently in progress in diabetic and non-diabetic patients with a clinical diagnosis of NAFLD (based on risk factors such as obesity and insulin resistance and the exclusion of other liver diseases) and raised liver tests[9]. In this 24-wk study, patients will be prescribed 4 g of betaine per day and after 4 wk will be randomized to either continue the same dose or increase to 8 g per day with the primary aim being comparing liver tests at weeks 12 compared to baseline using the paired *t* test. However, potential foreseeable limitations of this study include lack of histological confirmation or use of non-invasive markers of steatosis or fibrosis which have gained popularity in recent clinical trials as an effective substitute for liver biopsies. The duration of this study may also be of insufficient duration to determine any impact of betaine on the primary outcome. Finally, it remains unclear what the ideal dose of oral betaine should be for clinical trials in fatty

Table 1 Clinical trials of betaine for nonalcoholic steatohepatitis

Ref.	Patients (*n*)	Study design	Study duration	Pre- and post-treatment biopsies	Results
Miglio *et al*[5]	191	Double-blind, randomized, placebo-controlled.	8 wk	No biopsies	Improvement in liver test with betaine
Abdelmalek *et al*[6]	10	Polit, single-arm.	1 yr	Yes	Improvement in histology (3 did not complete study)
Abdelmalek *et al*[7]	35	Randomized, placebo-controlled.	1 yr	Yes	No biochemical or histological improvement but high drop-out rate
Mukherjee *et al*[8]	35	Prospective, single-arm cohort.	1 yr	Yes	Improvement in fibrosis in 62.9%

liver disease as discussed above as previous studies have used significantly larger doses. It therefore remains unclear if the study findings will change management of this disease due to limitations in study design but add to the literature without any meaningful impact.

Although several studies of betaine have been performed in pre-clinical (animal) studies, only SAM has been evaluated in patients with alcoholic liver disease[10,11]. In the first randomized, double-blind, placebo-controlled study, SAM improved survival and/or delayed listing for liver transplantation in 123 patients with alcoholic cirrhosis[12]. This may have been due to elevated levels of hepatic glutathione, an important antioxidant derived from homocysteine metabolism, which neutralizes free radicals and prevents cell injury. Elevated glutathione levels were also was reported in a second study of SAM in patient with alcoholic liver disease[13].

Although ASH and NASH are two different diseases, they share many features which include but are not limited to hepatic steatosis, mitochondrial dysfunction, endoplasmic reticular stress, gene dysregulation in lipid metabolism and excess proinflammatory cytokines[14]. Several of these functional and histological abnormalities are ameliorated with betaine supplementation in animal studies. For example, in well-established animal models of NAFLD, betaine supplementation attenuates the high-fat, diet-induced stress response in adipose tissue and improves both insulin sensitivity and adipokine synthesis as demonstrated by activation of hepatic adenosine monophosphate-activated protein kinase and inhibition of *de novo* lipogenesis[15]. In alcoholic liver disease where methionine synthetase activity is depressed, methionine and SAM levels are maintained by betaine supplementation which is activated by homocysteine in the presence of betaine homocysteine methionine methytransferase. An additional mechanism for betaine's protective effects in alcoholic liver disease may be due to nicotinamide adenine nucleotide synthesis by the methylation of norepinephrine to epinephrine by phenylethanolamine N methyl transferase. Although this study has not been performed in human subjects, the author suggested supplementing alcoholic beverages with betaine may prevent alcoholic liver disease[16].

CONCLUSION

The interest in betaine supplementation for ASH and NASH continues to evolve and should be re-evaluated in well-designed studies. Betaine has many properties that render it ideal for clinical trials for both of these conditions which are prevalent in both developed and developing countries where cost is a factor-betaine is naturally occurring, well tolerated and inexpensive. The plethora of clinical trials on NASH should not diminish the potential value of betaine which requires re-evaluation in a large, well-designed randomized trial of adequate duration. However, NASH is also prevalent in patients with human immune deficiency virus (HIV) disease who appear to have more aggressive disease than non-HIV positive patients but are often excluded from clinical trials due to their HIV status or concerns with drug interactions[17]. Betaine should be evaluated in this population of vulnerable patients and in those with alcoholic liver disease in whom histological improvement has even been described after liver transplantation[18].

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OPINION REVIEW

Management of an endoscopy center during the outbreak of COVID-19: Experience from West China Hospital

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Abstract

Since the outbreak of the coronavirus disease 2019 (COVID-19), various measures have been taken to protect against the infection. As droplet and contact transmission are the main routes of COVID-19 infection, endoscopy centers are considered to be high-risk areas for exposure to COVID-19. We have undertaken several countermeasures in our endoscopic center during the pandemic, and have gained significant experience in terms of prevention and control of COVID-19. We here present our experience and strategies adopted for preventing hospital infection in our endoscopy center during the COVID-19 pandemic. We describe our management of the environment, endoscope, patients, and medical staff, and our self-made masks.

Key Words: COVID-19; Endoscopy center; Management; Hygiene

Core Tip: This opinion review presents our experience and strategies adopted for preventing hospital infection in an endoscopy center located in Western China during the coronavirus disease 2019 (COVID-19) pandemic. We describe our three-level screening strategy and an innovative self-made gastroscope isolated mask. We provide valuable information for China and other countries suffering from the COVID-19 pandemic.

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INTRODUCTION

Since the outbreak of coronavirus disease 2019 (COVID-19) in December 2019, China and many other countries have tried several measures to protect against the spread of infection[1-3]. Although the COVID-19 pandemic in China has been mostly under control[4,5], the number of infected people in European and American countries is noticeably increasing[5,6]. Due to the main routes of droplet and contact transmission[7], endoscopy centers have a high risk of exposure to COVID-19. As the most prestigious endoscopy center in western China, a total of 7135 diagnostic endoscopies and 643 therapeutic endoscopies were performed in West China Hospital from March 1 to April 10, 2020, which included 124 cases of endoscopic retrograde cholangiopancreatography, 55 of endoscopic submucosal dissection, 207 of endoscopic mucosal resection, and 224 of endoscopic ultrasound. Compared with the same period last year, we reasonably arranged the endoscopy schedule and minimized unnecessary endoscopy (Table 1). There were no hospital infections in medical staff and patients. In the current review, we present our experience and strategies adopted for preventing hospital infection in our endoscopy center during the COVID-19 pandemic.

MANAGEMENT OF ENDOSCOPY CENTER

Disinfection of the environment and endoscopes

Endoscopy centers generally have no rooms with negative-pressure laminar flow. Virus transmission might occur because patients undergoing upper gastrointestinal endoscopy cannot wear a mask, and fecal fluid might flow during lower gastrointestinal endoscopy[8]. Thus, disinfection of the environment and endoscopes is important.

Disinfection of the environment: In our center, 500 ppm chlorine-containing disinfectant was applied 1 or 2 times/d for disinfecting the surface of equipment and the ground. Doorknobs and chairs were wiped and disinfected with 1000 ppm chlorine-containing disinfectant for 2 or 3 times/d. Secretions were removed immediately, and the areas wiped with 2000 ppm chlorine-containing disinfectant. Natural ventilation was performed continuously.

Disinfection of the operation room: Here, 1000–2000 ppm chlorine-containing disinfectant was applied at least 2 times/d for ground disinfection. Disinfection with 75% alcohol was used for consoles and monitors. Electrosurgical workstations and treatment beds were wiped with 75% alcohol before and after each procedure. Disposable bedspread and pillowcases were used for each patient. An automatic air disinfection machine was applied in the operating room twice daily (05:00–7:00 and 19:00–21:00 h). Continuous ventilation was conducted using a fresh air ventilation system during working hours.

Disinfection of the resuscitation room: Surfaces of monitors and other instruments were wiped with 75% alcohol for disinfection. Additionally, sofas were wiped and disinfected with 1000–2000 ppm chlorine-containing disinfectant for 1 or 2 times/d. Floors were mopped with 1000–2000 ppm chlorine-containing disinfectant for 1 or 2 times/d. An automatic air disinfection machine was used for air disinfection in the operation room twice daily (05:00–7:00 and 19:00–21:00 h). Continuous ventilation was performed using a fresh air ventilation system during working hours.

Disinfection of the office area: The doorknobs in office areas were wiped and disinfected with 75% alcohol for 2–4 times/d. The desktops, chairs and cabinet surfaces were wiped and disinfected with 1000 ppm chlorine-containing disinfectant. The floor was mopped and disinfected with 1000 ppm chlorine-containing disinfectant. Continuous ventilation was carried out using a fresh air ventilation system during working hours.

Disinfection of the endoscopes: After preprocessing, the endoscope was immersed into a container with 0.2%–0.35% peracetic acid for predisinfection. Instruments should also be predisinfected using the solution with 0.2%–0.35% peracetic acid solution. Then, the endoscope was placed in a special container, sealed, and transferred to a clean and disinfected room. Standard cleaning and disinfection procedures were carried out after 10 min. Finally, total ethanol perfusion with 75% alcohol and full sterilization with 0.2%–0.35% peracetic acid were done before the

Table 1 Endoscopy indigestive endoscopy center of West China Hospital during the COVID-19 pandemic

	March 1, 2019 –April 10, 2019	March 1, 2020-April 10, 2020
Routine gastroscopy	1414	616
Routine colonoscopy	903	438
Painless gastroscopy	4709	3325
Painless colonoscopy	3312	2462
Enteroscopy	24	30
Capsule gastroscopy	57	40
EUS	309	224
Diagnostic endoscopy	10728	7135
Removal of foreign body in UGI tract	109	70
Hemostasis of varicose veins	111	77
Hemostasis of non-varicose veins	25	19
Stent placement in UGI tract	1	4
Stent placement in LGI tract	2	6
Esophageal EMR	1	0
Gastric EMR	161	30
Colorectal EMR	569	177
Esophageal ESD	24	23
Gastric ESD	41	23
Colorectal ESD	14	9
POEM	5	0
ERCP	168	124
EUS-FNA	21	25
EUS + stent drainage	0	1
STER	8	7
ESTD	35	48
Therapeutic endoscopy	1295	643

UGI: Upper gastrointestinal; LGI: Lower gastrointestinal; EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection; POEM: Peroral endoscopic myotomy; ERCP: Endoscopic retrograde cholangiopancreatography; EUS: Endoscopic ultrasonography; EUS-FNA: Endoscopic ultrasound-guided fine-needle aspiration biopsy; STER: Submucosal tunneling endoscopic resection; ESTD: Endoscopic submucosal tunnel dissection; COVID-19: Coronavirus disease 2019.

application of endoscope to the next patient.

After daily cleaning and disinfection, the cleaning tank, rinsing tank, perfusion device, and cleaning brush were thoroughly cleaned and disinfected with 0.2%–0.35% peracetic acid.

Enhancing access management system

Three special channels with eye-catching signs were set up, for entrance and exit of medical staff as well as patients. Dedicated staff were appointed for managing these channels and recording related information.

Entrance and exit channels for medical staff: Measurement of medical staff's body temperature was undertaken before their entrance into a specific channel. In our center, staff without fever (< 37.3°C) were allowed to enter the endoscopy center, and protective equipment, such as disposable mask, hat, and surgical gown were provided. The body temperature of the staff was measured again when they left work.

Entrance channel for patients: Patients who underwent secondary screening (see below) were allowed to enter our endoscopy center. Family members had no access to the endoscopy center. Entrance was permitted for one family member only if the patient was in serious condition.

Exit channel for patients: Patients who received endoscopic intervention had to leave the endoscopy center through an exit channel. Patients' entrance was prohibited as well.

MANAGEMENT OF PATIENTS WITHOUT EMERGENCY

Prehospital management

Appointment scheduling for patients: Personal mobility and gathering might increase the risk of coronavirus transmission. Diagnostic appointment scheduling for asymptomatic patients without warning signs (*e.g.*, unexplained weight loss), and therapeutic plans for patients with mild illness (*e.g.*, small colorectal polyps) should be delayed. The appointment should be arranged according to the potential severity of illness. Priority should be given to patients who are old, frail, children, have a history of taking immunosuppressants, have diabetes, or to other immunocompromised patients. In our center, all patients were informed about the risk of virus infection, if they would like to receive endoscopy during the outbreak.

Patient survey and preparation prior to endoscopy: Dedicated staff contacted patients *via* telephone within 2 wk before their scheduled appointment, and inquired about the presence of the following symptoms and epidemiological history. Symptoms mainly included new-onset fever, cough, loss of appetite, and diarrhea. Epidemiological histories included residency in high-risk areas within 2 wk, and history of contact with COVID-19 patients and suspected cases within 2 wk. Patients with any of these symptoms and epidemiological history were referred to the fever clinic, and endoscopy was performed only after ruling out COVID-19 infection. More than 30 patients reported fever during telephone consultation, and they were ruled out for COVID-19 infection before undergoing endoscopy. Patients without these suspicious symptoms or epidemiological history also have to undergo chest computed tomography (CT) 3–5 d prior to endoscopy. Study showed that chest CT had a low rate of missed diagnosis of COVID-19 (3.9%, 2/51) and thus it can be used as a standard method for diagnosis of COVID-19 based on CT features and transformation rules[9]. Rapid diagnosis can control the potential spread early and optimize patient management. During the outbreak, a male patient with history of contact with people from Hubei Province was confirmed with COVID-19 after undergoing chest CT and following virus detection, although he declared suspicious symptoms during telephone inquiry. Our strategy aimed to prevent the transmission of the virus from such atypical patients.

Intrahospital management

We implemented a three-level screening strategy for patients who received endoscopy during the COVID-19 pandemic (Figure 1).

Primary screening in the gate of the main building: All patients had to wear face masks, and only patients without fever were allowed to enter the building. Dedicated staff rechecked the body temperature and investigated the epidemiological history of patients with fever. These patients were sent to the fever clinic outside the building, and were guided by the staff.

Secondary screening in the gate of endoscopy center: The body temperature, symptoms, epidemiological history, and the results of chest CT of each patient were recorded and checked. Patients without fever and epidemiological history, and with normal chest CT findings within 3–5 d were allowed to enter the endoscopy center for endoscopic operation after disinfection of their hands using alcohol-based hand rubs. With the gradual easing of the pandemic, the time requirement for chest CT can be extended to 2 wk before examination. Suspected patients were sent to the fever clinic for further examination, and guided by staff, while confirmed patients were quarantined in a special isolated ward.

Tertiary screening in the operating room: When patients arrived at the operating room of the endoscopy center, an endoscopist and a nurse rechecked the information

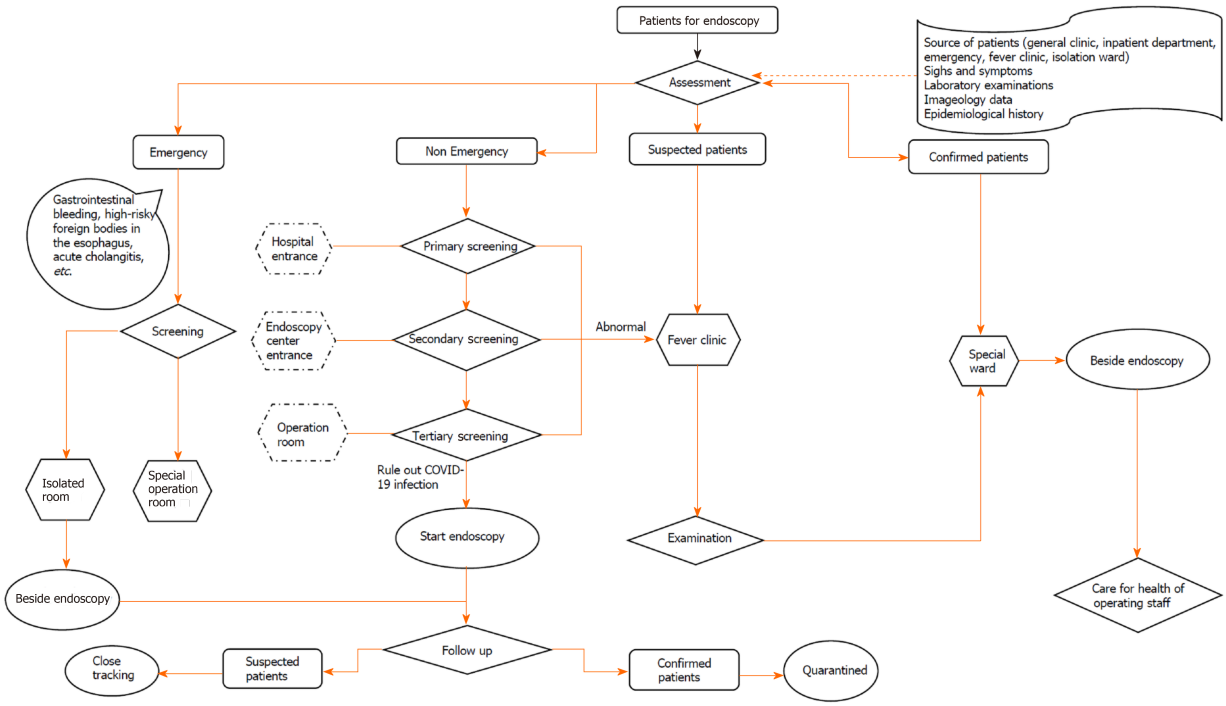

Figure 1 Workflow of digestive endoscopy during the COVID-19 pandemic. COVID-19: Coronavirus disease 2019.

recorded by the secondary screening. Suspected patients were sent to the fever clinic, and guided by the staff, while confirmed patients were quarantined in a special isolated ward. The endoscopic operation was then carried out as usual for unsuspected patients.

Posthospital management: Follow-up was conducted when patients left our center. Dedicated staff contacted the patients *via* telephone and recorded any abnormalities. If a patient was diagnosed with COVID-19 during the follow-up period, all the related medical staff had to stop working, and be quarantined at their respective homes for at least 2 wk. Related patients would be contacted and quarantined as well.

MANAGEMENT OF MEDICAL STAFF

The work plan was adjusted according to the number of appointed patients, and with minimized medical staff on duty. All endoscopists, nurses, and healthcare personnel were trained in terms of infection control, as well as utilizing personal protective equipment properly during endoscopy (Table 2). All staff had to daily report and record their symptoms and epidemiological history. Staff with fever or epidemiological history of exposure were referred to the fever clinic and were quarantined. Four medical staff of our endoscopy center were under home quarantine for 14 d due to confirmed cases in their community.

Before daily working

All medical staff had to change their personal clothes and wear a gown before entering the endoscopy center. They had to enter the main building through a channel different from patients after measuring their body temperature. Before meeting patients, medical staff had to perform hand hygiene, and wear a disposable surgical gown, with a face mask, hat, goggles, gloves, and protective shoe covers.

During daily working

The symptoms and epidemiological history of patients were rechecked before further intervention. Staff had to maintain a distance from each patient in the explanation step before starting endoscopic intervention. Hands had to be washed before and after contact with each patient, after contact with a potential source of infection, and before and after wearing and removing personal protective equipment, including gloves[10].

Table 2 Prevention and control measures for each location

Location	Occupational protection	Reserved supply	Environmental management			Screening and treatment		
			No special pollution	Special pollution	Air disinfection	Screening method	No abnormalities	Suspected patients
Reservation office	Long sleeve overalls; medical surgical mask; disposable medical cap; plastic gloves/nitrile gloves	Infrared temperature measuring gun Thermometer; 75% alcohol. Free hand-washing disinfection	Surface disinfection: 75% alcohol Floor disinfection: 500 mg/L chlorine-containing disinfectant Frequency: 1-2 times/d	Secretion pollution: 2000 mg/L chlorine-containing disinfectant Frequency: immediate	Continuous fresh air exchange	Ask about symptoms and signs and epidemiological history Temperature measurement	Make an appointment	Stop appointment Send to fever clinic of emergency department
Reception and guidance area	Long sleeve overalls; medical surgical mask; disposable medical cap; plastic gloves /nitrile gloves	Infrared temperature measuring gun Thermometer; 75% alcohol Free hand-washing disinfection	Surface disinfection: 75% alcohol Floor disinfection: 500 mg/L chlorine-containing disinfectant Frequency: 1-2 times/d	Secretion pollution: 2000 mg/L chlorine-containing disinfectant Frequency: immediate	Continuous fresh air exchange	Ask about symptoms and signs and epidemiological history Temperature measurement Fill in the preliminary screening form	Lead to secondary waiting area	Stop appointment Send to fever clinic of emergency department
Secondary waiting area	Long sleeve overalls; medical surgical mask; disposable medical cap; Plastic gloves/nitrile gloves; Patients and families wear masks all the time	Infrared temperature measuring gun Thermometer; 75% alcohol Free hand-washing disinfection	Surface disinfection: 500 mg/L chlorine-containing disinfectant; Floor disinfection: 500 mg/L chlorine-containing disinfectant; Frequency: 1-2 times/d; Doorknob and chairs disinfection: 1000 ppm chlorine-containing disinfectant (2 or 3 times/d)	Secretion pollution: 2000 mg/L chlorine-containing disinfectant; Frequency: Immediate	Continuous fresh air exchange	Check the preliminary screening table, symptoms and signs, and epidemiological historyTake temperature if necessary	Prepare endoscopic diagnosis and treatment	Stop diagnosis and treatment Send to fever clinic of emergency department
Examination room	Long sleeve overalls; disposable waterproof isolated clothing; Medical surgical face mask and glasses; Disposable medical cap; latex gloves; special shoes; The assistant is the same as the reservation office	Free hand-washing disinfection (per room)	Surface disinfection: 75% alcohol Floor disinfection: 1000–2000 mg/L chlorine-containing disinfectant Frequency: 2 or 3 times/d	Secretion/blood pollution: 2000 mg/L chlorine-containing disinfectant Frequency: Immediate	Continuous fresh air exchange Automatic air disinfector Time: 2 h Frequency: 2 times/d	Check the preliminary screening table, symptoms and signs, and epidemiological historyTake temperature if necessary	Endoscopic diagnosis and treatment	Stop diagnosis and treatment; Send to fever clinic of emergency department
Resuscitation room	Long sleeve overalls; medical surgical mask; disposable medical cap; plastic gloves/nitrile gloves	Free hand-washing disinfection (per bed)	Surface disinfection: 75% alcohol Floor and sofa disinfection: 1000–2000 mg/L chlorine-containing disinfectant Frequency: 1 or 2 times/d	Secretion/blood pollution: 2000 mg/L chlorine-containing disinfectant Frequency: immediate	Continuous fresh air exchange Automatic air disinfector Time: 2 h Frequency: 2 times/d	Make sure they wear the mask when the patient leaves the resuscitation room; Publicity and education for family members		
Disinfection room	Long sleeve overalls; Disposable waterproof isolated clothing; medical surgical face	Free hand-washing disinfection	Surface disinfection: 500 mg/L chlorine-containing disinfectant Floor disinfection: 500 mg/L chlorine-containing disinfectant	Secretion/blood pollution: 2000 mg/L chlorine-containing	Keep the storage cabinet ventilated, clean and dry	Suspend use of glutaraldehyde, and use peracetic acid for disinfection/sterilization		

	mask and glasses; disposable medical cap; latex gloves; special shoes		Frequency: 1 or 2 times/d	disinfectant Frequency: Immediate		
Office area	Personal clothes; Mask; All personnel are not allowed to come to the office with protective isolated clothing	Free hand-washing disinfection	Surface disinfection: 1000 mg/L chlorine-containing disinfectant Floor disinfection: 1000 mg/L chlorine-containing disinfectantFrequency: 2 or 3 times/d Doorknob disinfection: 75% alcohol (2–4 times/d)		Continuous fresh air exchange	Workers shall pack and seal the soiled bags in time
Staff for confirmed or suspected patients	Long sleeve overalls; disposable waterproof isolated clothing; medical protective clothing; protective face mask; protective glasses; N95 mask; protective shoe cover; double latex gloves	Free hand-washing disinfection; inside/outside the door of operation room	Surface disinfection: 2000 mg/L chlorine-containing disinfectant Floor disinfection: 2000 mg/L chlorine-containing disinfectant Frequency: Immediately after operation		Continuous fresh air exchange automatic air disinfector Time: 2 h/time Frequency: immediately after each operation	Operation room: Room 1; isolation corridor; staff: 2 doctors, 2 nurses and 2 anesthesiologists. All items prepared before operation, including environmental disinfection items. All remaining items shall be taken out after removing the outer package after the end of disinfection. All medical wastes shall be treated as hazardous wastes. After the final disinfection, the medical staff shall wait for 20 min for fresh air exchange, then remove the isolation protection facilities, turn on the sterilizer, after 30 min of disinfection, take off the mask and leave the inspection room. Disinfect the door handle again outside the door and have hand hygiene

Three levels of protection were required in case of exposure to respiratory secretions, such as tracheal intubation, airway care, and sputum aspiration for general patients, as well as during performing any endoscopic procedure on confirmed or suspected COVID-19 patients[11]. The tissue samples obtained during endoscopy were stored in a fixed area and the endoscopy report was provided to avoid cross-infection.

After daily working

Medical staff took off their disposable items in the exit channel, and put them in the medical waste bin. To save materials, we did not discard all protective materials after each operation. We changed gloves and disinfected hands after each operation. Other protective equipment was not be replaced if there was no secretions or splashes. A seven-step hand-washing method was applied. Body temperature was recorded before leaving the endoscopy center. Staff changed from their gown to personal clothes, and put the gown in a specific tub. When off duty, staff stayed indoors and cooperated with the epidemic prevention management of the community. The manager of the endoscopy center communicated with the hospital management team closely and regularly, monitored the outbreak closely, and changed the plans quickly to deliver sustainable and effective endoscopy services.

SELF-MADE ISOLATED MASK FOR UPPER GASTROINTESTINAL ENDOSCOPY

Based on the concept that patients with air-borne infectious disease should be isolated in negative pressure wards in order to prevent infection[8], a fully-enclosed "isolated mask" for patients undergoing upper gastrointestinal endoscopy was developed. The mask provided both oxygen inhalation and negative pressure (for suction of patients' expulsion from mouth and nose) (Figures 2 and 3). The end of the mask cannula could be freely closed with a unidirectional flap at the entrance, which was fixed on the inside of the cannula to prevent patients' expulsion from overflowing. There were two separate windows on the left and right sides of the mask bulge. One window was reserved for the oxygen tube, and the other was used for negative pressure suction.

The size of the window aperture was the same as the diameter of the tube, and it was sealed tightly. After the mask was inflated, it closely fitted the patient's face to avoid expulsion leakage. The oxygen inhalation flow rate was adjusted to 6–8 L/min. The suction tube was connected to the central negative pressure system and the aspirated liquid (gas) was continuously removed to the hospital waste liquid (gas) treatment center. We have applied this isolated mask to 162 patients for respiratory isolation during upper gastrointestinal endoscopy (Figure 4). The gastroscopy process was smooth and the vital signs of the patients remained stable during and after the operation. No hospital infection occurred in our endoscopy center.

CONCLUSION

Our endoscopy center achieved effective epidemic prevention and undertook much diagnostic and therapeutic work during the COVID-19 pandemic. Several factors influenced the outcome of this management. First, experienced personnel led the overall situation and laid the foundation for success. We established a special prevention and control group at the beginning of the pandemic: The director as the team leader, the head nurse as the deputy team leader, and the infection control nurse and anesthetic medical team leader as the backbone. We quickly started our emergency procedures and adjusted the working mode in time: Delayed ordinary endoscopy, reduced the flow and aggregation of people, and kept special channels for patients with emergencies. In addition, we implemented a three-level screening mechanism and strengthened personnel access management. We improved the cleaning and disinfection of endoscopes and the environment. We formulated standards for pandemic protection in our endoscopy center, strictly implemented the health supervision of employees, and trained all staff in the use of personal protective equipment. Finally, although not used in large quantities, our innovative isolated mask played an important role. Our strategies may provide a comprehensive management for endoscopy centers during the COVID-19 pandemic.

As an infectious disease mainly transmitted by respiratory tract and contact, COVID-19 has its own special characters. The COVID-19 pandemic has attracted unprecedented global concern, which needs global unity. COVID-19 is transmitted from respiratory secretions, feces, and contaminated environmental surfaces. In addition, the virus is spread not only by patients with symptoms but also by asymptomatic individuals[9]. However, control of the pandemic can be realized through reasonable prevention and management. Other infectious diseases with the same or similar route of transmission can be managed by this method in medical environments with high risk of exposure, such as endoscopy centers. Some parts of this strategy (like three-level screening) can also be applied in community hospitals, which generally provide no endoscopic procedures for patients.

In conclusion, further protection of patients and medical staff is required during the outbreak of COVID-19 or other infectious diseases. We share our experience to provide valuable information for China and other countries that are suffering from the COVID-19 pandemic.

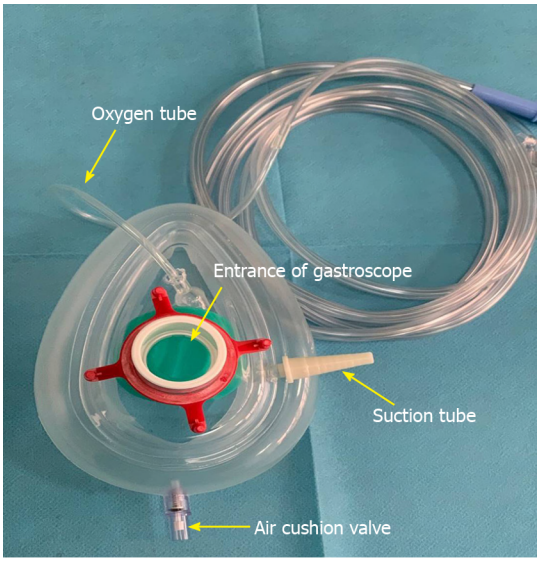

Figure 2 Outside surface of the mask.

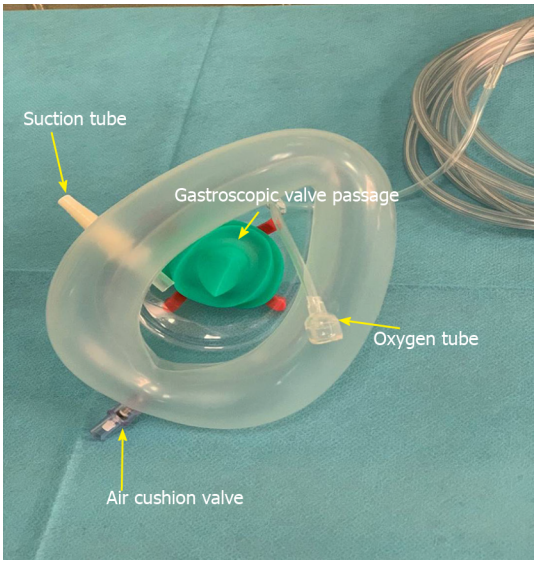

Figure 3 Inside surface of the mask.

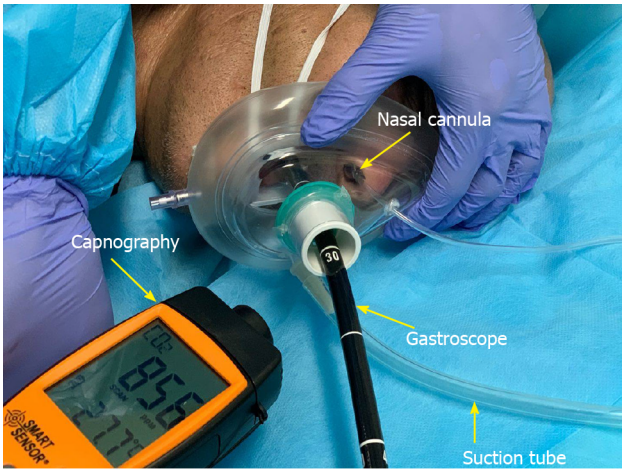

Figure 4 Use of mask during endoscopy.

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REVIEW

Molecular mechanisms of viral hepatitis induced hepatocellular carcinoma

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Abstract

Chronic infection with viral hepatitis affects half a billion individuals worldwide and can lead to cirrhosis, cancer, and liver failure. Liver cancer is the third leading cause of cancer-associated mortality, of which hepatocellular carcinoma (HCC) represents 90% of all primary liver cancers. Solid tumors like HCC are complex and have heterogeneous tumor genomic profiles contributing to complexity in diagnosis and management. Chronic infection with hepatitis B virus (HBV), hepatitis delta virus (HDV), and hepatitis C virus (HCV) are the greatest etiological risk factors for HCC. Due to the significant role of chronic viral infection in HCC development, it is important to investigate direct (viral associated) and indirect (immune-associated) mechanisms involved in the pathogenesis of HCC. Common mechanisms used by HBV, HCV, and HDV that drive hepatocarcinogenesis include persistent liver inflammation with an impaired antiviral immune response, immune and viral protein-mediated oxidative stress, and deregulation of cellular signaling pathways by viral proteins. DNA integration to promote genome instability is a feature of HBV infection, and metabolic reprogramming leading to steatosis is driven by HCV infection. The current review aims to provide a brief overview of HBV, HCV and HDV molecular biology, and highlight specific viral-associated oncogenic mechanisms and common molecular pathways deregulated in HCC, and current as well as emerging treatments for HCC.

Key Words: Chronic viral infection; Hallmarks of cancer; Hepatocellular carcinoma; Hepatitis B virus; Hepatitis C virus; Hepatitis delta virus co-infection; Molecular mechanisms; Viral hepatitis

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Core Tip: Hepatocellular carcinoma (HCC) is a dreaded complication of viral infection with hepatitis B virus and/or hepatitis delta virus and hepatitis C virus. Many of the direct and indirect molecular mechanisms used by these viruses to co-opt the liver microenvironment for persistence also disrupt cell cycle pathways. Indirectly, the immune system has a major role in the contribution of HCC in the context of viral hepatitis, but direct viral mechanisms also create a pro-tumorigenic environment.

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INTRODUCTION

Epidemiology of viral hepatitis associated hepatocellular carcinoma

Liver cancer is the third leading cause of cancer-associated mortality (781631 people/year), despite being ranked seventh on global incidence (841080 people/year)[1]. Approximately 12% of all cancer cases globally arise from chronic infections with bloodborne oncogenic viral pathogens including hepatitis B virus (HBV), hepatitis C virus (HCV), and hepatitis delta virus (HDV)[2]. Although incidence in the majority of cancers has decreased, primary liver cancer incidence is the fastest-growing cancer with regards to incidence and mortality[3]. Hepatocellular carcinoma (HCC) represents 90% of all liver cancer cases and the risk factors are well defined: Viral infection with HBV, (54% of all HCCs) and/or HCV (31% of all HCCs), cirrhosis (80% of all HCCs), high alcohol consumption, obesity, genetic disorders such as hemochromatosis, exposure to aflatoxins, sex (male) and older age (50+)[4-7].

Virus-induced HCC is present worldwide, however, there are considerable differences in populations that develop HBV or HDV induced HCC *vs* HCV induced HCC. HBV and HDV associated HCC is more common in low and middle-human development index countries, while HCV induced HCC is more common in high and very high-human development index[2]. Chronic hepatitis B (CHB) infection affects around 257 million people worldwide, of which 48-60 million people are co-infected with HDV and an estimated 2.6 million are co-infected with HCV[8-10]. Exposure to infected blood/bodily fluids is the primary mode of transmission for HBV and HBV/HDV, with majority of exposures occurring from mother to child during birth or early years of life. Unvaccinated neonates and children who have been exposed to HBV have > 95% risk of developing chronic disease, while infection during adulthood results in < 2% chance of developing chronic disease[11]. HBV/HDV co-infection have the highest mortality rate (20%) associated with any viral hepatitis infection and most severe liver disease (*i.e.* acute liver failure, cirrhosis within 5 years, and HCC within 10 years)[10,12,13]. HCV has established chronic infection in 70 million people primarily through horizontal blood-borne transmission routes such as intravenous drug use, needle pricks, unscreened blood transfusions, and high-risk sexual practices[11]. In comparison to HBV or HCV mono-infection, individuals who are co-infected with HBV/HCV have increased rates of HCC development. Overall, viral etiologies represent approximately 80% of all HCC related cases, highlighting the importance of investigating the role of these viruses in the development of liver cancer.

Preventative measures against HBV and HDV induced liver cancer include birth-dose vaccinations, hepatitis B immunoglobulin treatment for children born to infected mothers as well as treatment of mothers with high HBV viral load with nucleos(t)ide inhibitors in the third trimester[14]. For those individuals who are already chronic carriers of HBV/HDV, there is no virological cure; however, treatment with nucleos(t)ide analogs can lower the risk of HCC development[15]. There is no protective vaccine available for HCV, but there are effective direct-acting antivirals that can cure > 90% of chronic carriers. Those who have a sustained virological response from direct-acting antiviral treatment have a significantly lower risk of HCC development if cirrhosis is absent[16]. Although there are treatment options to lower the risk of HCC in those who have chronic viral hepatitis infection, globally many individuals are unaware of their status, lack access to testing, and effective treatment.

In this review article, we discuss the molecular biology of HBV, HCV, and HDV, common features associated with virus-induced cancers, viral oncogenic mechanisms leading to HCC relating to the hallmarks of cancer, common molecular pathways deregulated in HCC, and current as well as emerging treatments for HCC.

OVERVIEW OF VIRAL LIFE CYCLES

Hepatitis B virus life cycle

The HBV is a member of the *Hepadnaviridae* family, which has a cellular tropism for hepatocytes, but has also been detected in extra-hepatic reservoirs such as the lymphoid cells (*i.e.* peripheral blood mononuclear cells)[17-20]. HBV has a compact 3.2 kb partially double-stranded relaxed circular DNA genome (rcDNA) containing four overlapping open reading frames: Pre-S/S, X, P, and pre-C/C, which are under the transcription control of the pre-S1 promoter, pre-S2/S promoter, enhancer I/X and enhancer II/basal core promoter[21]. The viral protein products include three surface proteins (large/pre-S1, middle/pre-S2, and small/S - also known as HBsAg), the core antigen (HBcAg), the excreted "e" antigen (HBeAg), the viral polymerase (which has reverse transcriptase, DNA polymerase, and RNaseH activity), and the X protein (HBx) that plays an essential role in HBV pathogenesis and viral transcription[21]. Upon viral attachment of the envelope HBV preS1 protein to the sodium taurocholate co-transporting polypeptide receptor, the virus is endocytosed (Figure 1). The nucleocapsid is transported *via* microtubules from the cytoplasm to the nucleus where the rcDNA is converted to covalently closed circular DNA (cccDNA)[22,23]. The cccDNA associates with histone and non-histone proteins which form a viral minichromosome that persists in the hepatocyte to serve as the template for transcription of pregenomic RNA (pgRNA) and subgenomic RNAs by host RNA polymerase II[24]. The exported pgRNA and subgenomic transcripts are translated to produce the core protein, viral envelope surface proteins, HBeAg, polymerase, and X proteins. In addition, the pgRNA transcript is packaged by the capsid proteins and reverse transcribed by the viral polymerase into rcDNA. The newly packaged rcDNA can either localize back to the nucleus to replenish the cellular cccDNA population or gain their coat through the endoplasmic reticulum (ER)/Golgi and proceed to bud out of the to infect other cells[25]. Current nucleos(t)ide antivirals target the viral reverse transcriptase to produce aberrant transcripts that cannot produce infectious virions. Additional details about the lifecycle and host-transcription factors/proteins required for HBV replication are included in our previous article by Turton *et al*[26].

Hepatitis Delta virus life cycle

HDV is the smallest human infecting virus and the sole member of the *Deltavirus* genus[27]. HDV is characterized as a "satellite" or "defective" virus as it is dependent on HBV co-infection for viral assembly and persistence. HDV has an approximate 1.7 kb circular, single-stranded, negative-sense RNA genome that encodes for a single protein of two isoforms: The small and large delta antigens (S-HDAg and L-HDAg, respectively)[28]. Viral entry (Figure 2) occurs similarly to HBV due to HDV's co-opted use of the envelope HBsAg protein[29]. Following viral entry, HDV uncoats in the cytoplasm and the ribonucleoprotein complex consisting of the HDV viral genome and HDAg complex is imported into the nucleus[30]. Rolling-circle replication occurs in the nucleolus using the host RNA polymerase II to produce antigenomic positive sense HDV RNA that serves as a template for genomic HDV RNA synthesis and protein production[31]. The antigenome can be edited by host protein adenosine deaminase acting on RNA 1 (ADAR1) to change adenine to inosine in the UAG stop-codon to produce the L-HDAg. The edited and non-edited antigenomes are then linearized by the HDV associated ribozyme, exported to the cytoplasm, and translated to HDV antigens. The non-edited transcript produces S-HDAg (24 kDa) and the transcript modified by ADAR1 produces the L-HDAg (27 kDa)[32]. Following extensive post-translational modifications, the viral antigens associate with the HDV RNA in the cytoplasm to form the ribonucleoprotein complex. The ribonucleoprotein is trafficked through the ER and Golgi apparatus where it co-opts the HBsAg envelope produced by HBV, and then buds out of the cell[33].

Hepatitis C virus life cycle

The HCV is part of the *Flaviviridae* family and the genus *Hepacivirus*. HCV is an enveloped, positive-sense, 9.6 kb single-stranded RNA virus with highly conserved 5′ and 3′ untranslated regions[34]. HCV primarily infects hepatocytes due to the

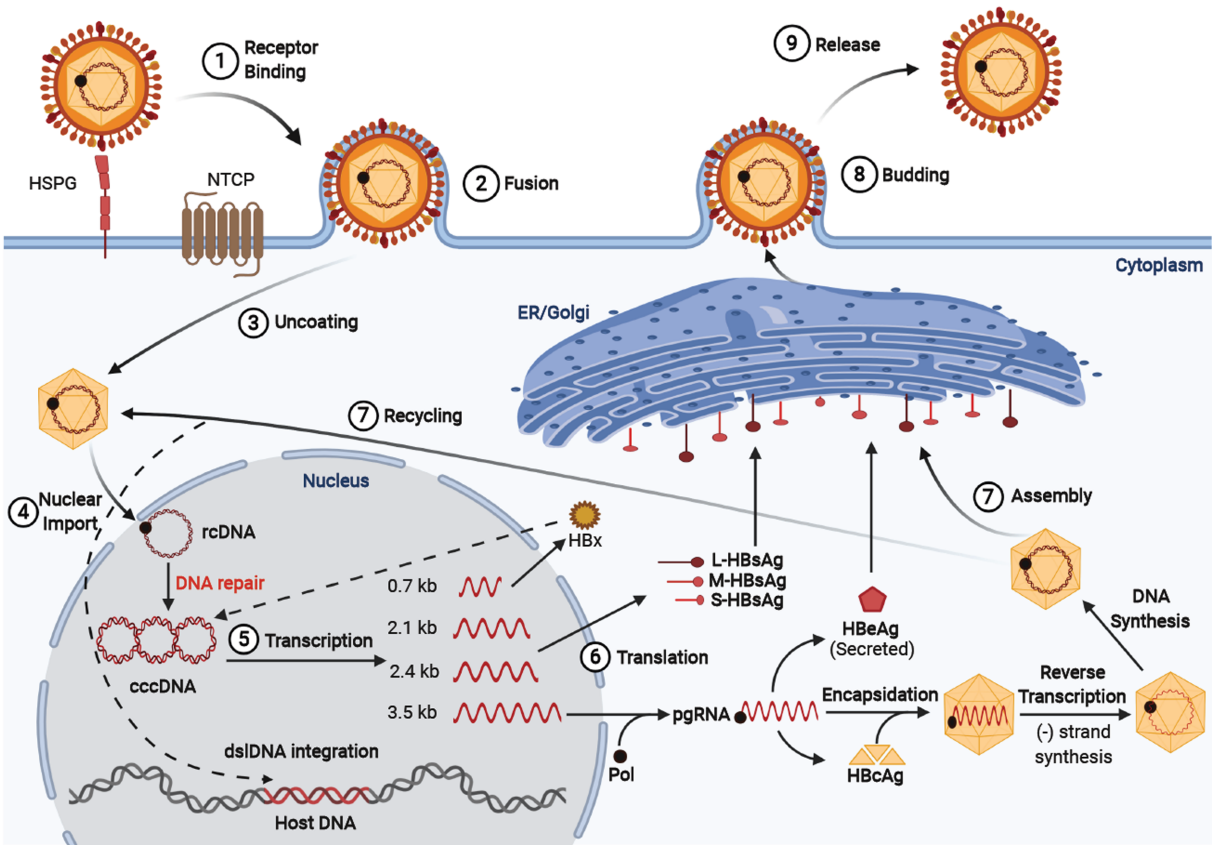

Figure 1 Hepatitis B virus life cycle. Viral entry is mediated by low-affinity binding of the Pre-S1 protein to the heparin sulfate proteoglycan receptor, followed by binding to the sodium-taurocholate co-transporting polypeptide to facilitate entry. The nucleocapsid is transported from the cytoplasm to the nucleus where the relaxed circular DNA (rcDNA) genome is converted into the persistent covalently closed circular DNA (cccDNA) form. Viral mRNA is then transcribed from the cccDNA genome and translated at the rough endoplasmic reticulum. The greater than genome length pregenomic RNA is transported to the cytoplasm, encapsidated by the hepatitis B virus core protein and reverse transcribed by the hepatitis B virus polymerase to produce rcDNA or double-stranded linear DNA. The core particles can then obtain their envelope proteins at the endoplasmic reticulum to be excreted out of the cell, or the core particles containing double-stranded linear DNA can relocate into the nucleus and integrate into the host genome, and the rcDNA can be recycled intracellularly to replenish the cccDNA pool.

expression of essential entry receptors and liver-specific cellular host factors (miRNA-122) required for viral replication[35]. However, extrahepatic manifestations have been observed in peripheral blood mononuclear cells, epithelial cells, kidneys and in the peripheral nervous system[36]. Through complex mechanisms, HCV particles interact with several receptors (see[37] for details) to induce conformational changes and proceeds to enter the cell (Figure 3) *via* clathrin-mediated endocytosis[37]. Endosomal acidification causes the fusion of the viral envelope to the endosome membrane, disassociation of the viral core, and release of the HCV RNA genome into the cytoplasm[37]. In the ER the viral RNA is replicated and translated from a single open reading frame using the 5′ untranslated regions internal ribosomal entry site. The translated product is an approximately 3000 amino acid polyprotein precursor that is cleaved by host and viral proteases to form ten proteins[38]. There are three structural proteins - core, E1 and E2 - and seven non-structural proteins p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B that have roles in polyprotein cleavage, viral replication, assembly, and release[39]. More recently, two isoforms of the core protein, known as the "mini-core" were discovered to be translated from an alternative open reading frame at amino acids 70 and 91 which preserve the c-terminal portion of the mature p21 core nucleocapsid but lack the N-terminus. The function of these mini-core proteins has yet to be elucidated, however, mutations in amino acid positions 70 and 91 are associated with increased risk of HCC, insulin resistance, and failure of interferon treatments[40,41]. Following viral replication and protein translation, the core protein assembles in the ER on a lipid droplet and recruits HCV viral RNA which is subsequently encapsidated[42]. The viral nucleocapsid is then processed through the ER-lumen and Golgi apparatus for maturation and the HCV particle associated with very low-density lipoproteins are released from the plasma membrane[38,43,44].

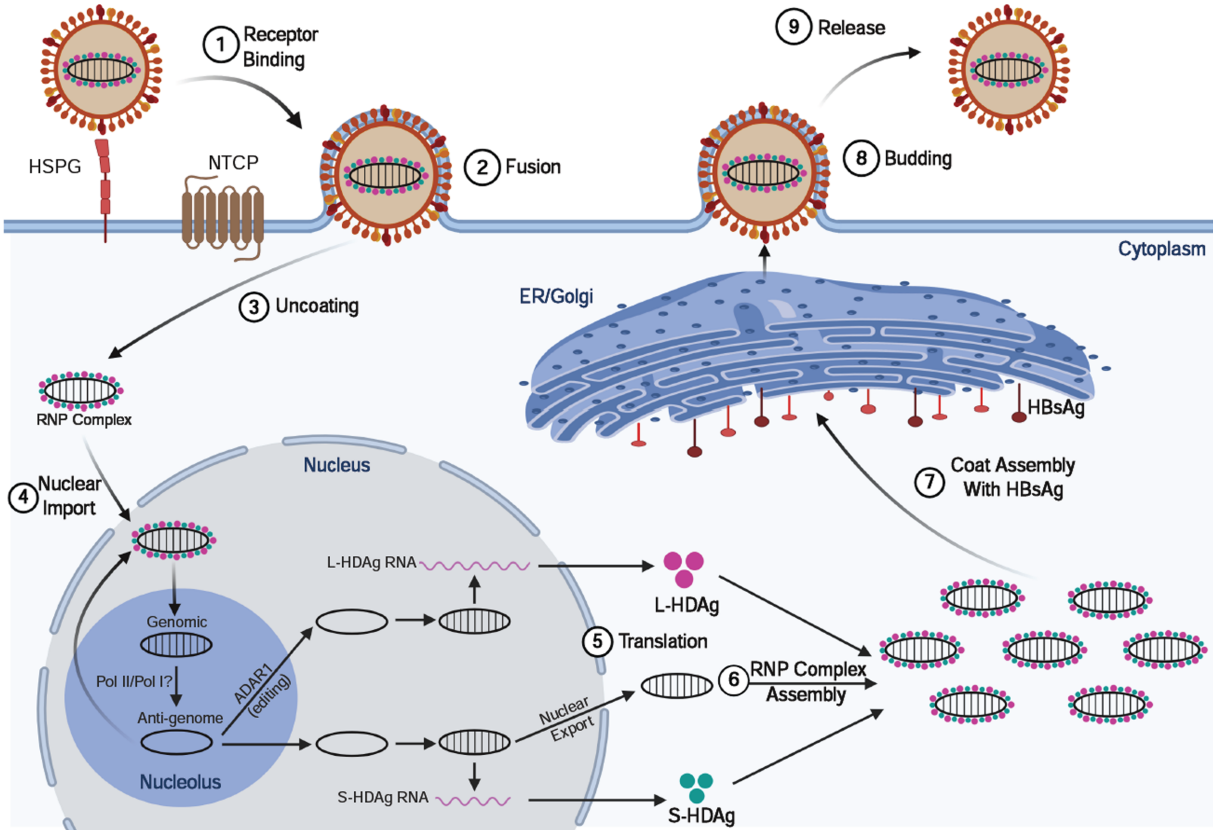

Figure 2 Hepatitis delta virus lifecycle. Viral entry is mediated (like hepatitis B virus) by low-affinity binding of the Pre-S1 protein to the heparin sulfate proteoglycan receptor, followed by binding to the sodium-taurocholate co-transporting polypeptide to facilitate entry. Following uncoating, the ribonucleoprotein (RNP) complex consisting of negative-sense single-stranded RNA genome plus the small and large hepatitis delta virus (HDV) antigens (L-HDAg/S-HDAg) are transported to the nucleus. Within the nucleolus, HDV RNA is replicated using a double rolling circle amplification to form the positive-sense anti-genomic RNA and more genomic RNA. From the amplification process, the genomic RNA is transported out of the nucleolus and into the nucleus where it can be transcribed to produce the S-HDAg transcript or undergo A to I editing by ADARI to produce the L-HDAg RNA. Once the RNA transcripts are exported out of the nucleus, translation machinery produces the S-HDAg and L-HDAg which associate with the genomic HDV RNA to produce the RNP complex. The RNP complex passes through the endoplasmic reticulum and Golgi apparatus to obtain its coat and are then released out of the cell to infect neighboring hepatocytes. ER: Endoplasmic reticulum; NTCP: Sodium-taurocholate co-transporting polypeptide; HSPG: Heparin sulfate proteoglycan receptor; RNP: Ribonucleoprotein.

Overview of mechanisms driving HCC development with infection by HBV, HCV, HDV

HBV, HCV, and HDV use several mechanisms to co-opt the infected cells which may unintentionally lead to HCC development. Commonly used mechanisms between all three viruses include: (1) Persistent liver inflammation and immune-mediated oxidative stress damage from a chronic viral infection; (2) Intracellular oxidative stress damage induced by viral proteins; and (3) Deregulation of cell signaling pathways by viral proteins (*e.g*. HBx, L-HDAg, S-HDAg, HCV core, NS3, and NS5A/B). HBV is the only hepatotropic DNA virus that also uses viral DNA integration to induce genome instability, which can lead to the creation of fusion gene products, and altered expression of oncogenes or tumor suppressors. In addition, HCV facilitates metabolic reprogramming leading to steatosis, which aids in the progression of fibrosis and HCC.

General traits of oncogenic viruses

There are several viral traits that are common to human oncogenic viruses[45,46]: (1) Oncoviruses are ubiquitous in the environment and infection alone is not sufficient for cancer development. Although chronic infection with HBV/HCV/HDV results in higher rates of HCC development, not all persistently infected individuals develop liver cancer. Thus, this observation would suggest that the viruses by themselves are insufficient for cancer development. (2) Virally induced cancers are biological accidents as tumor formation is not an intentional outcome of viral infection. (3) Viral cancers appear in the context of persistent infections and occur many years to decades after initial exposure. Hepatic viruses have co-evolved with their hosts and hence,

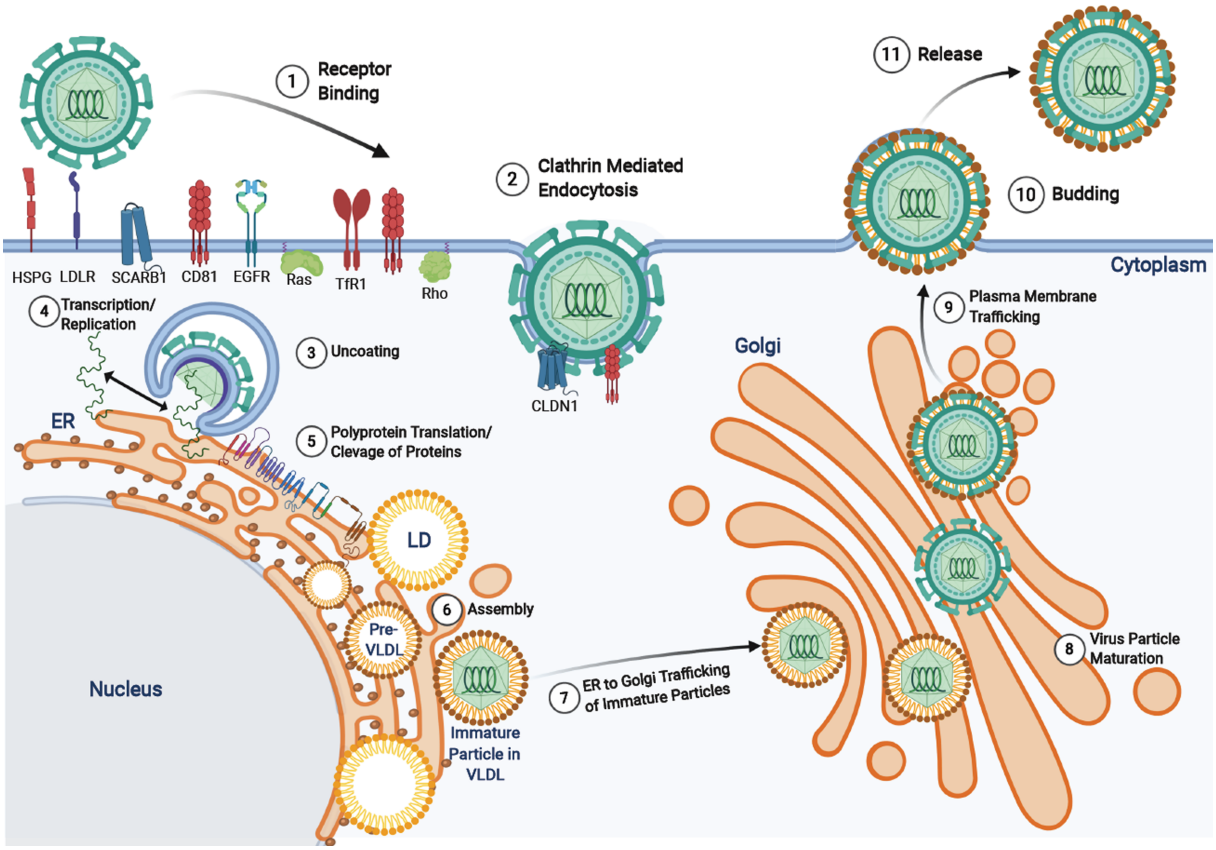

Figure 3 Hepatitis C virus life cycle. Hepatitis C virus entry is facilitated by a variety of receptors and signaling pathways (described in[37]). Upon viral entry, the positive sense RNA genome is released into the cytoplasm from endosomal acidification. The viral RNA undergoes replication and translation at the rough endoplasmic reticulum to produce a single polyprotein chain at the endoplasmic reticulum membrane that is cleaved by viral and host proteases into 10 different viral proteins (structural and non-structural). The virus particles are assembled on lipid droplets and associate with very-low-density lipoproteins which mature at the Golgi apparatus and are released *via* the secretory pathway. HSPG: Heparin sulfate proteoglycan receptor; LD: Lipid droplets; VLDL: Very-low-density lipoproteins.

have evolved effective immune evasion strategies to establish long-term infection such as expression of viral proteins that interfere with innate interferon responses, inflammation, and adaptive immunity. (4) Most viral remnants within a tumor are non-infectious and tumors are non-permissive for viral replication. Active virion production is typically absent in transformed tumor cells. (5) All virally induced cancers have non-infectious co-factors that influence tumorigenesis. Host factors such as age, sex, genetics, environmental factors, and immunodeficiencies are associated with viral hepatitis-related HCC. (6) The immune system can play a deleterious or protective role, with some virus-associated cancers increasing with immunosuppression and others appearing during chronic inflammation. In the context of viral hepatitis induced HCC, the host antiviral immune response is unable to eliminate virally infected cells and instead causes immune-mediated damage. This phenomenon is evident in chronic infections where bouts of repeated hepatitis caused by the inflammation-necrosis-proliferation cycle leads to the production of reactive oxygen species (ROS) that promote genetic mutations, fibrosis, cirrhosis, and HCC.

The features of oncogenic viruses described above reflect the multifactored nature of virus-induced hepatocarcinogenesis. Human oncovirus infection alone is insufficient to directly drive cancer and viral infection provides only a portion of the oncogenic alterations. The combination of viral factors and other factors (*i.e.* host, environment, time) is generally required for the development of cancer[45]. The hallmarks of cancer outline developed by Hanahan and Weinberg[47] deconstruct the specifics of cellular deregulation into factors that contribute to cancer development[47]. This outline also explains the reliance on time progression to accumulate oncogenic mutations and the multistep nature of acquiring various hallmarks that eventually lead to cancer. By applying this system to viral-induced cancers, we can better understand how alterations in cellular processes induced by hepatitis viruses contribute to HCC development (depicted in Figure 4 and Figure 5).

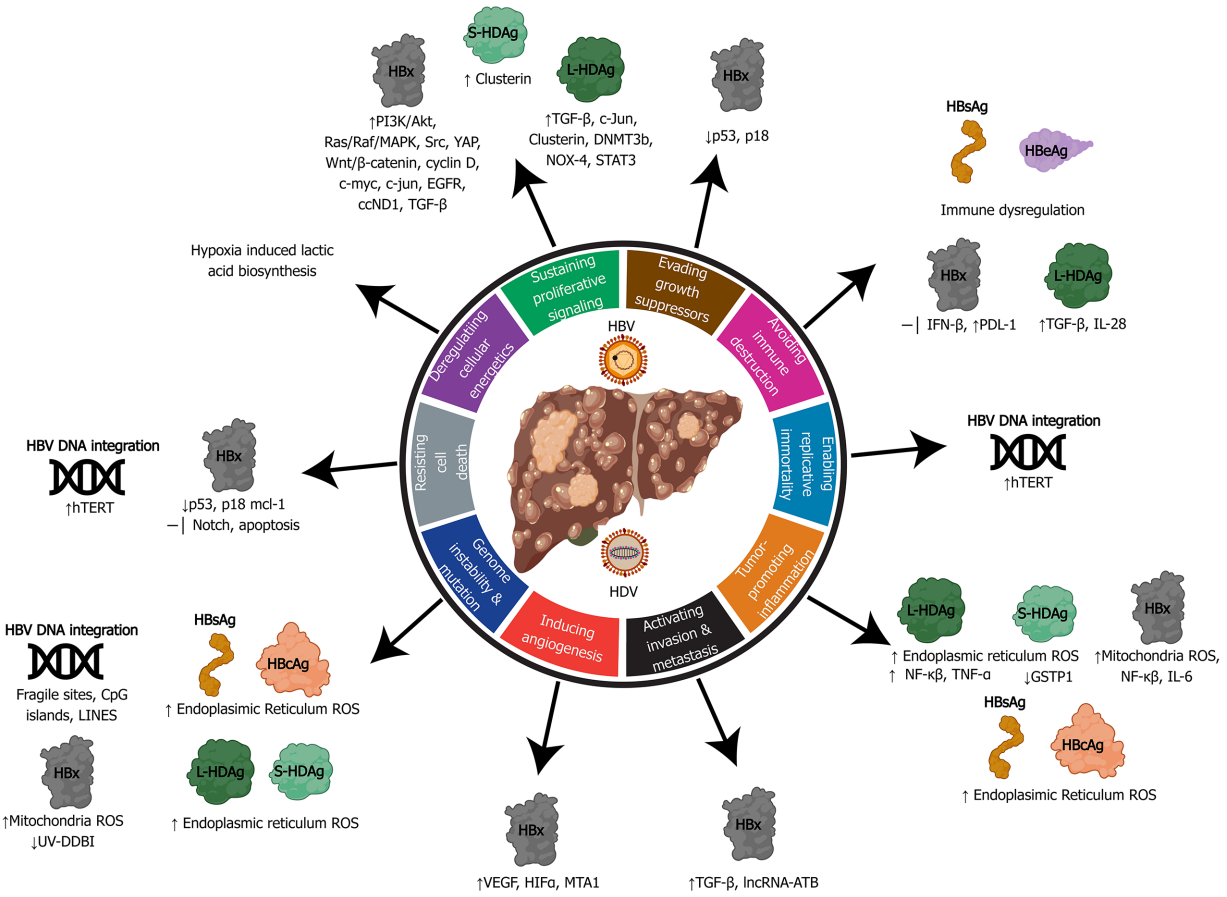

Figure 4 Relating the hallmarks of cancer to the molecular mechanisms of hepatitis B virus and delta virus hepatocarcinogenesis. Hepatitis B virus can activate all ten hallmarks of cancer using viral proteins (HBx, HBsAg, HBeAg, HBcAg) and DNA integration. Hepatitis delta virus has been linked to four hallmarks, primarily through molecular mechanisms manipulated by the large and small hepatitis delta virus antigens (L-HDAg and S-HDAg). HBV: Hepatitis B virus; HDV: Hepatitis delta virus; ER: Endoplasmic reticulum; ROS: Reactive oxygen species.

Specific viral factors affecting HCC development

Viral genotypes vary across the globe and play an important role in virus treatment response and assessing HCC risk. HBV has ten genotypes (A-J) which have a genetic divergence of > 8%. The HBV genotypes associated with the highest risk of HCC development are genotype C > B > F > D > A[7,48]. Some studies suggest that individuals infected with either HBV genotype B or C that have T1762/A1764 basal core promoter mutations have a higher risk of HCC development in younger individuals (< 50 years old) without cirrhosis[49]. In HBV genotype C infections, mutations/deletions in the preS region, enhancer II at position C1653T, and/or T173V in the basal core promoter can predict the development of HCC in 80% of cases[50]. Moreover, genotypes A and B have a better response to peg-IFN-α therapies, while there are no genotypic preferences for nucleos(t)ide analog treatments[51].

HCV has 6 major genotypes (1-6) that have a genetic divergence of 31%-35%. With HCV, studies linking genotype to the risk of developing HCC have inconsistent findings[7]. However more recently, a large cohort study of United States veteran concluded that HCV genotype 3 infections had an 80% higher risk of HCC development compared to genotype 1[52]. In a southeast Asian cohort, HCV genotype 6 is also associated with an increased is of HCC development[53]. With currently approved direct-acting antiviral treatments for all HCV genotypes, sustained virological response is observed in > 90% of treated individuals and reduces HCC risk in individuals without cirrhosis[54].

There are eight different HDV genotypes (1-8), which have a large genetic divergence ranging from 20%-40%[55]. There has not been a significant amount of research conducted to elucidate the effects of HDV genotypes on clinical outcomes. One study concluded that genotype 1 is associated with worse clinical disease including HCC than genotype 2[56]. Moreover, clinical outcomes of the disease are potentially regulated by both HBV and HDV genotypes. Due to the reliance of HDV on HBV co-infection, the only treatment option for HDV infection is peg-IFN-α, which

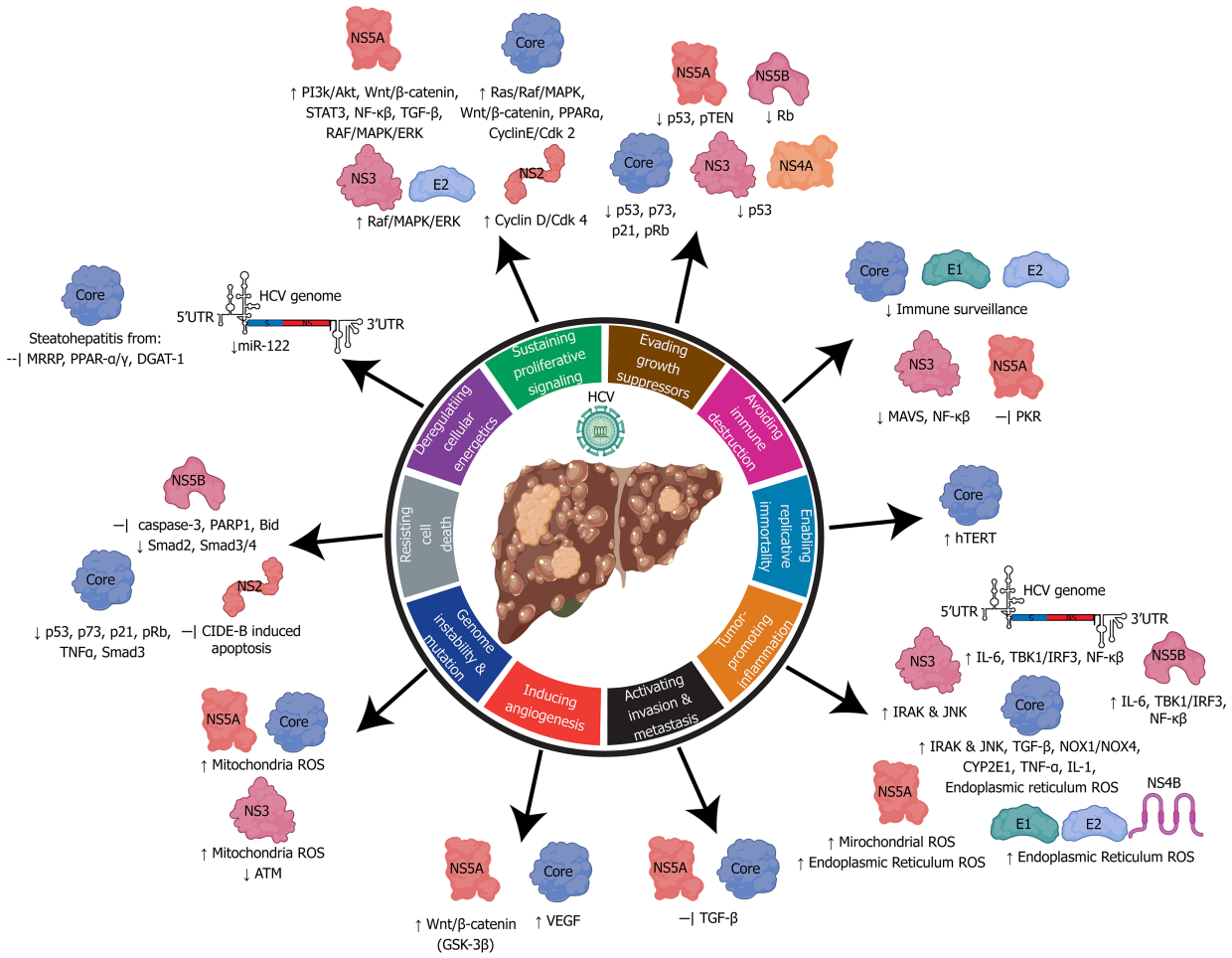

Figure 5 Relating the hallmarks of cancer to the molecular mechanisms of hepatitis C virus hepatocarcinogenesis. Hepatitis C virus uses its RNA genome and many viral associated structural and non-structural proteins to alter cellular pathways to influence all ten hallmarks of cancer. HCV: Hepatitis C virus.

is poorly tolerated and has < 30% response rate, highlighting the urgent need for improved therapies[57].

CHRONIC INFLAMMATION-MEDIATED BY VIRAL HEPATITIS

Non-resolving inflammation is a hallmark of cancer that significantly contributes to the development and progression of HCC[47]. Approximately 80% of HCC cases arise from hepatocyte injury and chronic inflammation resulting in cirrhosis[6,58]. HCC in chronic hepatitis B, C, or HBV/HDV co-infection patients occurs in the presence of cirrhosis[59,60]. In contrast, 10%-20% of HBV-related HCC can occur in the absence of cirrhosis and liver inflammation[61]. Under normal circumstances, the innate and adaptive immune responses are activated during an infection or tissue injury and immune cells are recruited to fight against the pathogen and induce wound healing. Following the elimination of the pathogen *via* cytolytic and non-cytolytic mechanisms, the damaged tissue is repaired through the wound-healing process[62]. However, the persistence of the inflammatory stimuli (*e.g.* chronic viral infection) or dysregulation of the immune regulatory mechanisms prevents complete wound-healing and causes non-resolving inflammation that may lead to liver complications resulting in autoimmunity, fibrosis, cirrhosis, metaplasia and/or tumor growth[62].

There are five clinical phases of chronic hepatitis B infection (Figure 6A) from the 2019 AASLD guidelines[63]: $HBeAg^+$ chronic infection, $HBeAg^+$ chronic hepatitis, $HBeAg^-$ chronic infection, $HBeAg^-$ chronic hepatitis, and a functional cure ($HBsAg^-$). Each clinical phase is defined by a host immune response with respect to HBV viral activity. During the initial HBeAg+ chronic infection phase the host is immune response has a poorly activated HBV-specific CD8+ T-cell response[64]. Transition to the chronic hepatitis phase is characterized by increased activation of the adaptive

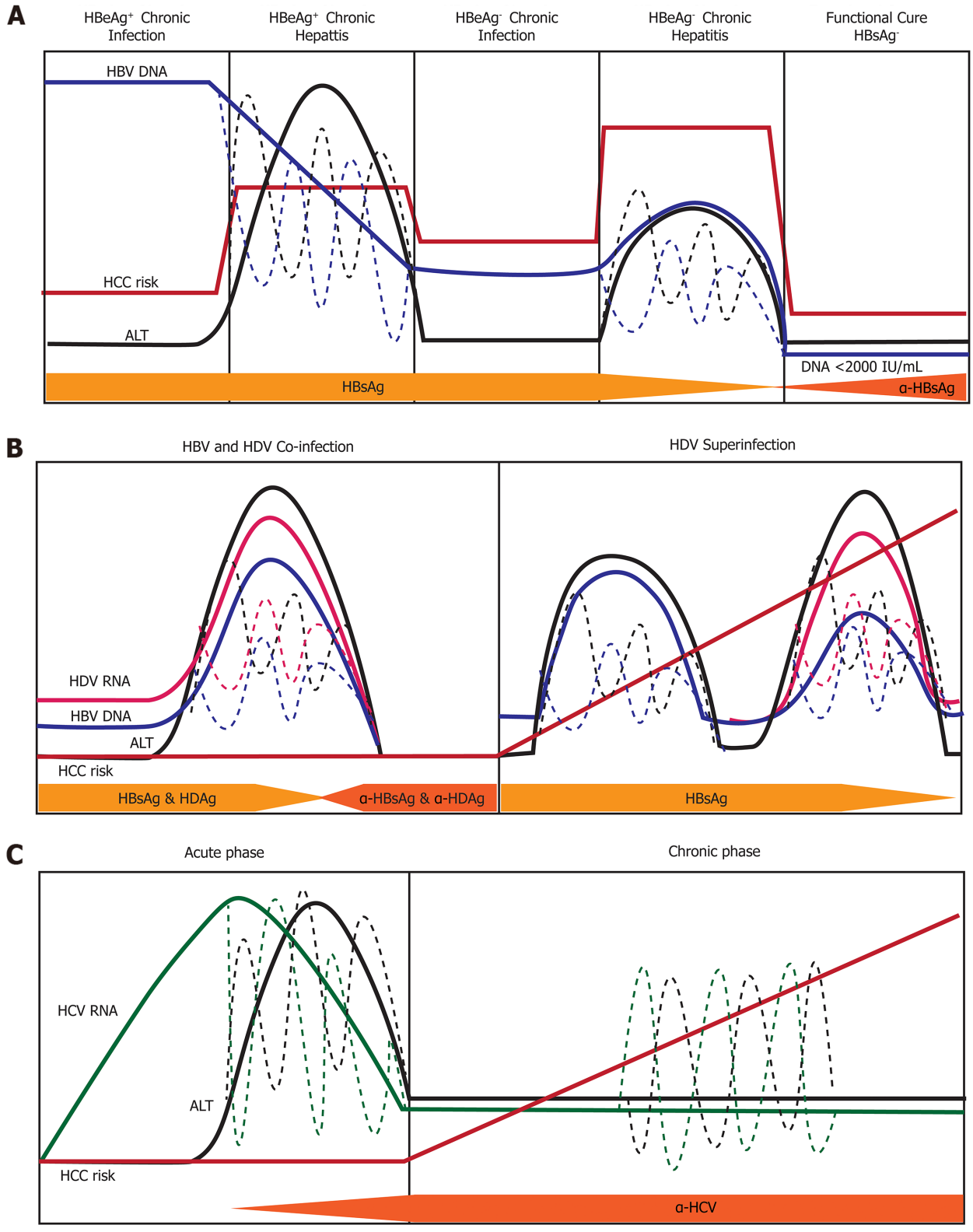

Figure 6 Natural history of infection with hepatitis B, delta, or C virus. Variations in hepatitis B virus (HBV) DNA, hepatitis C virus RNA, hepatitis delta virus (HDV) RNA, and ALT levels indicated by dashed lines. A: Natural history of chronic Hepatitis B virus infection. There are five phases of infection HBeAg$^+$ chronic infection, HBeAg$^+$ chronic hepatitis, HBeAg$^-$ chronic infection, and HBeAg$^-$ phase. Each clinical phase is defined by a host immune response with respect to HBV viral activity; B: Natural history of HDV infection in either HBV co-infection or HDV superinfection when the individual is a chronic carrier of HBV; and C: Natural history of Hepatitis C virus infection. There are two main phases of infection acute infection and chronic infection. HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; HDV: Hepatitis delta virus; HCV: Hepatitis C virus.

immune response (*e.g*., HBV-specific CD8$^+$ T-cells, pro-inflammatory cytokines) which causes decreased HBV DNA levels, liver inflammation, and variable/progressive liver fibrosis. Failure to completely clear HBV in the HBeAg$^+$ chronic infection phase results in prolonged over-active immune cell-mediated damage that leads to rapid liver disease progression. Immune-mediated liver damage is facilitated by natural killer cells and T-cells through the release of ROS and proinflammatory cytokines which causes bouts of necroinflammation, hepatocyte regeneration/healing and remodeling of the liver microenvironment[65,66]. Constant necroinflammation and failed wound healing responses lead to prolonged oxidative stress exposure which can promote the rapid development of fibrosis, cirrhosis, and cell transformation (epigenetic alterations, oncogenic mutations, telomere shortening, and genomic instability)[67,68]. The 5-year cumulative HCC risk for CHB patients with cirrhosis ranges from 9.7%-15.5%[69]. However, 20% of HCC caused by HBV does not require liver cirrhosis, indicating there are other intrinsic viral associated factors that are responsible for transforming hepatocytes.

HDV infection occurs either in a co-infection model with HBV or as a superinfection from horizontal transmission in individuals with CHB (Figure 6B). The mechanisms used by HDV to modulate the immune system are different from that expressed by HBV and HCV due to the consistent presence of co-infection. The natural history of chronic HDV infection is also dynamic and can be characterized as[70]: (1) Suppressed HBV replication and active HDV replication with high ALT; (2) Slightly decreased HDV replication and HBV reactivation with moderate ALT; and (3) Late-stage disease where cirrhosis and HCC are caused by either HBV/HDV or remission resulting in a reduction of both HBV and HDV viral load. During initial infection, HDV evades IFN-α-mediated innate immune responses to promote cell survival and viral persistence[71]. Under normal cellular conditions, double-stranded RNA induces expression IFN-α which binds to the IFN receptor-associated JAK kinase tyrosine kinase-2(tyr-2). Dimerization of the tyr-2 receptor activates a JAK/STAT signaling cascade to produce innate antiviral proteins: myxovirus resistance A, 2′,5′-oligoadenylate synthase, and dsRNA-activated protein kinase[72]. HDV blocks phosphorylation of tyr-2 to prevent downstream signaling and impairs phosphorylation activity and nuclear accumulation of STAT1/STAT2[71].

The superinfection of HDV in patients with CHB has the most severe liver disease outcome, partially due to the pre-existing liver damage caused by HBV infection[73]. Moreover, superinfection with HDV leads to HBV viral load suppression through mechanisms that are not thoroughly understood[74]. Recognition of MHC-1 HDV antigens on infected cells by CD8$^+$ T-cells mediates cellular killing. Released viral antigens are endocytosed by Kupffer cells (liver resident macrophages), B-lymphocytes, and dendritic cells and presented to CD4$^+$ helper T-cells *via* MHC-II receptors. Clonal expansion of CD4$^+$T-cells releases IL-2, IL-10, and IFN-γ cytokines which stimulate immune-mediated killing of HDV infected cells, severe liver necrosis and progressive liver disease[75].

Infection from the HCV is usually acquired from horizontal transmission in adulthood, where 75%-80% of people develop a chronic infection from viral persistence[11]. Chronic hepatitis C (CHC) infected individuals have mild liver inflammation (Figure 6C), stable HCV RNA titers, and liver disease that progresses especially in the presence of other risk factors (age, male, obesity, diabetes alcohol, HIV or HBV co-infection)[76]. HCV infection activates intrinsic type I and III IFN responses which induces transcription of innate-antiviral IFN stimulated genes[77]. Adaptive viral-specific CD8+/CD4+ T-cells and natural killer cells facilitate the release of pro-inflammatory cytokines and growth factors while destroying of HCV infected hepatocytes by promoting the inflammation-necrosis-proliferation cycle[78]. Immune-mediated damage produces large amounts of ROS mediated DNA damage, lipid peroxidation, epigenetic modifications, mitochondrial alteration, senescence, and chromosomal translocation that lead to hepatocyte transformation[79]. Immune failure to remove all HCV infected cells causes the selection of viral escape mutants within a carrier population. These escape mutants prevent stimulation of CD4+/CD8+ T-cell responses, and aid in viral immune evasion, chronic infection, loss of immune regulation, and promotion of HCV-mediated HCC[80,81]. Moreover, persistent liver inflammation caused by immune cells over decades of infection can lead to the development of fibrosis, cirrhosis, and HCC. Approximately 10-20% of CHC patients develop cirrhosis in 20-30 years in the absence of treatment for hepatitis C, indicating the high risk for HCC development[82].

Cirrhosis is a major risk factor for HCC development

The liver is made up of approximately 80% parenchymal cells (*i.e.*, hepatocytes) and 20% non-parenchymal cells (*e.g.*, sinusoidal endothelial cells, hepatic stellate cells, and Kupffer cells)[83]. Infection with viral hepatitis primarily targets the large population of hepatocytes, leading to production of ROS. Release of ROS and pro-inflammatory cytokines by Kupffer cells/hepatocytes activate neighboring stellate cells and liver sinusoidal endothelial cells which are key players in the development of fibrosis[84,85] (Figure 7). Stellate cells and fibroblasts enhance collagen synthesis and alter the extracellular matrix which lead to remodeling of the liver microenvironment[86].

Progressive inflammation and fibrosis pave the way for disease progression to cirrhosis which is the largest risk factor for HCC development (Figure 7). Cirrhosis is irreversible and often individuals are asymptomatic, which makes diagnosis and treatment difficult[87]. Those who develop severe symptoms of cirrhosis, are likely to have advanced liver disease and HCC. This is especially problematic for the populations who are unaware of their infection status with HBV, HCV, and/or HDV because they are unable to seek treatment intervention to lower the risk of developing cirrhosis. During cirrhosis, altered blood flow can lead to a hypoxic environment for hepatocytes leading to altered molecular signaling and increased oxidative damage[88]. Cells within the context of cirrhosis have experienced a multitude of changes from inflammation mediated damage, repair, and regeneration. The hypoxic environment in the liver during cirrhosis can select for altered oncogenic cells and promote angiogenesis. For a comprehensive review of molecular mechanisms of host factors driving the progression of liver cirrhosis to HCC see (Fridland *et al*[88] and Kanda *et al*[89]).

HBV-SPECIFIC INDUCTION OF HCC

HBV DNA integration

Although HBV uses reverse transcription for replication, unlike retroviruses, integration is not an essential step in the virus lifecycle and does not produce replication-competent virus[90]. During reverse transcription of the pgRNA, partially double-stranded rcDNA is formed 90% of the time[17]. The rcDNA is the genetic material that can be used to replenish the cccDNA pool and produce viable virions that can proceed to infect new hepatocytes. For 10% of cases, the reverse transcription process does not produce rcDNA and instead synthesizes double-stranded linear DNA (dslDNA)[17]. The HBV dslDNA can also be present in virions and repaired to produce cccDNA with a 16nt insertion that cannot produce pgRNA (unless it reverts to wild type cccDNA *via* homologous recombination)[91]. Integration of dslDNA is reported to occur in 1 of approximately 10^5-10^6 infected hepatocytes, and has been observed to occur early in infection (children as young as 5 mo old), and in patients who have acute HBV, CHB, and HCC[23,92]. The currently accepted mechanisms for HBV integration driving HCC include (reviewed by Tu *et al*[17]): (1) Chromosomal instability from HBV integrated DNA; (2) Insertional mutagenesis in proto-oncogenes and tumor suppressors; and (3) Expression of mutant HBV proteins from integration[17].

A key hallmark of cancer is genome instability. Hepatitis B virus can induce genome instability through viral integration into the host genome to cause cellular transformation (Figure 4). In non-HCC patients, HBV integration sites are randomly distributed through the genome and do not contain enriched sequence mutations. However, in CHB-HCC patients, HBV integration can be enriched in certain areas to cause chromosomal instability through integration near fragile sites: Intergenic regions, repetitive regions (*e.g.*, LINEs), short interspaced nuclear elements, simple repeats, CpG islands, and telomeres[93]. Chromosomal rearrangements and gene copy number variations also contribute to chromosomal instability and are present in the majority of CHB associated HCC[94].

Next-generation sequencing studies that compare HBV integration sites between tumor and matched non-tumor tissues have found that HCC tumors generally have a greater number of integration events and increased integration frequency in coding or promoter regions. In non-tumorous HBV infected hepatocytes, recurrent integration in driver genes can promote hepatocyte clonal expansion[17]. In 10%-15% of HCC cases, recurrent integration of the enhancer II/core HBV promoter in/near telomerase reverse transcriptase (TERT) or myeloid/lymphoid or mixed-lineage leukemia 4 genes causes upregulation of these oncogenes[95,96]. The upregulation of these genes has been observed in early and late tumor development, which may indicate that integration in these genes may aid in cell transformation and HCC progression.

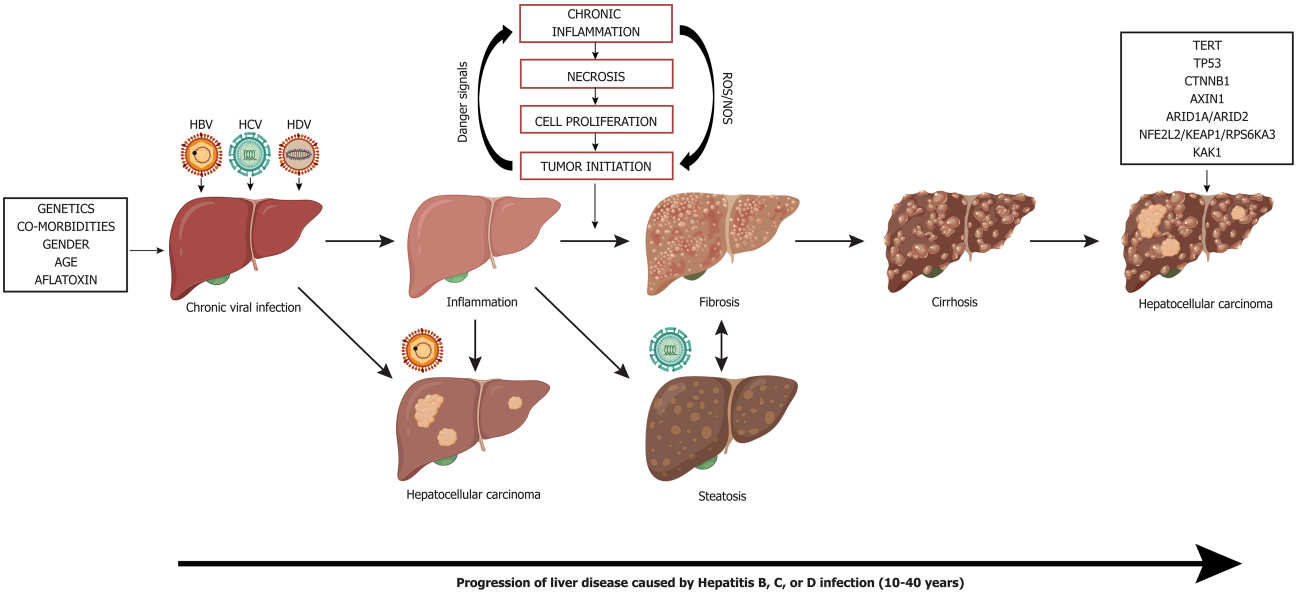

Figure 7 Liver disease progression to hepatocellular carcinoma from chronic viral hepatitis infection. Genetics, co-morbidities, gender, age, and aflatoxin exposure influence liver disease progression along with chronic viral infection with hepatitis B, C, and/or delta virus. Cirrhosis is the greatest risk factor for development of hepatocellular carcinoma, however, hepatocellular carcinoma in the context of chronic Hepatitis B virus infection can occur in the absence of cirrhosis. Chronic hepatitis C infection can lead to steatohepatitis, which can accelerate fibrosis and cirrhosis. Superinfection with Hepatitis delta virus in individuals who have chronic Hepatitis B virus infection creates an accelerated disease course leading to liver failure and/or hepatocellular carcinoma. Many driver mutations (telomerase reverse transcriptase, TP53, CTNNB1, AXIN1, ARID1A/ARID2, NFE2L2/KEAP1/RPS6KA3, KAK1) can occur as liver disease progresses to hepatocellular carcinoma and can lead to accelerated disease progression. TERT: Telomerase reverse transcriptase; HBV: Hepatitis B virus; HDV: Hepatitis delta virus; HCV: Hepatitis C virus.

Integration of HBV dslDNA can lead to the persistent expression of mutant and truncated HBsAg, HBcAg and HBx proteins. High expression rates of these normal and mutated proteins are associated with ER and mitochondrial stress responses which can increase the risk of HCC[97]. These mutants have also been observed to stimulate hepatocyte expansion and may provide a proliferative advantage. In animal models, over-expression of mutant HBsAg and HBx show precancerous liver lesions and HCC[98]. Moreover, expression of C-terminal truncated HBx protein from integrated HBV induces stem-cell-like properties, cell transformation, tumor invasion, and inhibition of apoptosis[17,99].

Deregulation of cellular pathways by HBx protein

The HBx protein (17 kDa) plays various roles in the HBV lifecycle and HCC development (Figure 4)[100]. HBx does not directly bind to DNA, instead, it interacts with other proteins to cause promiscuous transactivation of viral and cellular genes[101]. There are four main mechanisms used by HBx that contribute to HCC[102]: (1) Integration of HBx gene into the hepatocyte genome promoting genetic instability (Figure 4); (2) Interaction with the mitochondrial and other cellular proteins to induce oxidative stress; (3) Activation of cell survival signaling pathways and inactivation of tumor-suppressors; and (4) HBx induced epigenetic modifications such as DNA methylation, histone acetylation, and microRNA expression.

HBx modulates proto-oncogenic signaling pathways that are involved in inflammation and proliferation: Mitogen-activated protein kinase (MAPK)/Ras/Raf/c-Jun, NF-κβ, JAK-STAT, protein kinase C, Src, survivin and PI3K cascades[101,103]. HBx has also been proposed to activate the Wnt/β-catenin pathway through the binding of Antigen presenting cells protein or inactivation of GSK-3β through Extracellular signal regulated kinase activation. These mechanisms result in the accumulation of β-catenin and increased transcription of pro-angiogenic/metastatic factors[104,105]. HBx promotes genome instability through inhibition of UV-induced DNA damage repair pathways and S-phase progression by binding to UV-DDB1[106]. Hypoxic cirrhotic nodules expressing HBx promotes survival and growth through transcriptional activation/stabilization of HIF1α, which activates transcription of Ang-2 and vascular endothelial growth factor to promote angiogenesis and metastasis[107]. HBx also upregulates matrix metalloproteinases that digest fibrous capsules in tumors resulting in increased epithelial-mesenchymal transition (EMT) and metastasis[108]. HBx can also trans-activate cAMP-response element binding protein

response element genes and Yes-associated protein, which are often over-expressed in HCC[109]. The tumor suppressor p53 can also be bound by HBx in the cytoplasm and prevent p53 nuclear localization. Binding of p53 by HBx causes deregulation of cell-cycle checkpoints, inhibition of p53 dependent apoptosis and DNA-repair pathways. Loss of p53 activity leads to genome instability and the deregulation of tumor suppressors[47].

HBx is an epigenetic regulator of DNA hyper or hypomethylation in proto-oncogenes and tumor suppressors, respectively[110]. Viral-induced upregulation of DNMTs causes aberrant hypermethylation of CpG islands in tumor suppressor, leading to gene silencing[111]. In one study, 82% of HCC tumors had at least one tumor suppressor gene inactivated by hypermethylation, indicating the important role of epigenetic modifications in cancer development[112]. HBx protein increases the transcription of methyl catalase DNMT1, which hypermethylates the tumor suppressor gene E-cadherin and INK4A[113]. Loss of INK4A leads to loss of cell-cycle regulation, and loss of E-cadherin promotes epithelial to mesenchymal transition which promotes invasion and metastasis. HBx has also shown to promote histone acetylation and deacetylation to alter the expression of cancer-related genes, microRNAs, and non-coding RNAs. Increased levels of miR-29a, miR143, miR-148a, and miR-602 by HBx promotes upregulation of genes involved in angiogenesis and metastasis[114]. There are several miRNAs that are downregulated by HBx, one of the most important being miR-122 a liver-specific miRNA that has an anti-tumorigenic role[115]. HBx also induces expression of long non-coding RNAs: LINE1 which upregulates Wnt/B-Catenin (promoting invasion and metastasis), HULC, UCA1 which inhibit tumor suppressors p18 and p27 (promoting G1/S cell cycle transition), and DBH-AS1 which activates extracellular signal-regulated kinase (ERK)/p38/MAPK (anti-apoptosis)[114].

HBV proteins mediate intracellular oxidative stress

Individuals with CHB exhibit 1.5-4 times higher levels of oxidative stress (8-oxoguanine DNA products, lipid peroxidation, oxidation of proteins, decreased levels of anti-oxidant enzyme glutathione and higher oxidative forms) in the liver and plasma/sera compared to HBV negative individuals[116,117]. Extracellular oxidative stress can be immune mediated through the expression of pro-inflammatory cytokines or the release of ROS from cellular destruction. Intrinsic oxidative stress in the ER and mitochondria can be mediated by HBV associated proteins HBsAg, HBcAg, and HBx (Figure 4)[118]. These HBV associated proteins can be expressed from integrated HBV DNA or from the cccDNA minichromosome.

During the HBV lifecycle, secretory proteins such as the HBsAg and HBeAg are folded and assembled in the ER and transported through the Golgi[119]. High expression levels of secretory proteins or mutant HBV proteins that are misfolded can accumulate in the ER and cause activation of an unfolded protein response (UPR)[120]. The UPR induces inflammation, tissue damage from cell death, regeneration, and fibrosis (Figure 7). The wild-type and mutant LHBsAg and mutant HBcAg induce oxidative stress through protein accumulation in the ER membrane causing an UPR[121]. Excess LHBsAg leads to the blockage of HBsAg secretion, while mutant LHBsAg leads to ER stress, which may induce DNA damage and genomic instability[121-123]. A study of a Korean cohort with CHB genotype C suggested mutations in the HBcAg could upregulate ER stress resulting in ROS, increased pro-inflammatory cytokines, and increased intracellular Ca^{2+}[124]. Activation of the UPR response by HBsAg and HBcAg causes release of hydrogen peroxide and calcium ions into the cytoplasm, enhancing ROS production[121]. HBV infection also reduces anti-oxidative stress response pathways (*e.g.* Nrf2/ARE, catalase, and HO-1)[125]. Moreover, HBsAg is able to promote cell transformation through immune dysregulation, upregulation of survival signaling pathways, activation of transcription factors (NF-kB, AP-1, STAT3), increased mutations through the generation of free-radicals, cell-cycle deregulation, release of pro-inflammatory cytokines, and activation of stellate cells[67,126,127].

The HBx protein can localize into several cellular compartments (mitochondria, cytoplasm, and nucleus) to aid in various roles including transcription, cell-cycle progression, protein-degradation, apoptosis, and genetic instability[100]. Localization of HBx on the outer mitochondrial membrane, causes reduced expression and activity of respiratory complex proteins I, II, IV and V in the electron transport. Reduced cellular respiration results in altered mitochondrial function, increased production of superoxide anions, and 8-oxoguanine DNA products[118,128,129].

HDV-SPECIFIC INDUCTION OF HCC

HDV can indirectly mediate hepatocarcinogenesis through innate immune response modifications, induction of adaptive immune responses, epigenetic changes, lncRNA modifications and ROS production (Figure 4). The L-HDAg has an important role in facilitating many of these mechanisms through interaction with signaling pathways involved in pro-growth/survival, apoptosis, and wound healing[130,131]. Activation of the transforming growth factor β (TGF-β) and AP-1 pathways by L-HDAg binding of Smad3, STAT3, and c-jun promotes EMT, fibrosis, and cell-transformation[131,132].

HDV is also able to promote oxidative stress in the ER through L-HDAg's interaction with NOX-4[133]. Activation of the NOX4 pathway causes the release of ROS which can activate STAT3 and NF-κβ signaling[133]. The L-HDAg can also promote pro-inflammatory NF-κβ activity through stimulation of TNF-α. The S-HDAg can directly bind to glutathione S-transferase P1 mRNA causing downregulation in expression, increased ROS, and apoptosis[134]. Moreover, epigenetic modifications such as histone H3 acetylation by small and large HDAg enhances clusterin gene expression[128]. Increased levels of clusterin and histone acetylation aid in HDV infected cell survival and are upregulated in cancerous cells[135,136].

HCV-SPECIFIC INDUCTION OF HCC

Viral protein-mediated oxidative stress

Similar to HBV, individuals with chronic HCV infection experience significant decreases in antioxidant enzymes, and two to seven logs increase in liver and blood oxidative stress[137,138]. Prolonged oxidative stress results in increased levels of free oxygen radicals, DNA adduct formation (*e.g.* 8-oxoG), protein adducts, and lipid peroxidation[116,139,140].

In the ER, oxidative stress is mediated by HCV core, E1, E2, NS4B, and NS5A proteins (Figure 5). Viral glycoproteins E1/E2, and non-structural protein NS4B induce the unfolded protein response in the ER, which causes calcium release and production of hydrogen peroxide[141,142]. NS5A facilitates calcium uptake in the mitochondria and ER, causing release of hydrogen peroxide and organelle dysfunction[143]. HCV core-mediated binding of the mitochondria activates the mitochondrial calcium uniporter facilitating the uptake of ER released calcium ions[144]. Subsequently, an influx of calcium into the mitochondria directly effects the electron transport chain and leads to increased ROS production[144,145].

Enhanced expression of TGF-β1 from HCV core and NS5A upregulates the production of Nicotinamide adenine dinucleotide phosphate oxidases NOX1/NOX4 and cytochrome p450 2E1 oxidase (CYP2E1)[146,147]. CYP2E1 aids in metabolism of ethanol and drugs with the release of superoxide and hydrogen peroxide by-products[148]. Although CYP2E1 has an important metabolic role, high levels of expression induced by HCV core and NS5A lead to increased levels of ROS by-product accumulation[146,149]. In the context of HCV induced fibrosis, CYP2E1 expression levels are also increased which may imply that ROS have an important role in liver damage[150]. NOX1 expression and localization to the nuclear membrane is stimulated shortly after HCV infection and promotes release of superoxide ions into the cytoplasm[151]. NOX4 can be found on the nuclear or the ER membranes, which release hydrogen peroxide into the cytoplasm or nucleus promoting direct DNA damage[151].

Hepatic steatosis

Steatohepatitis is characterized by the presence of excess triglycerides in hepatocytes. HCV promotes steatohepatitis through enhancing lipogenesis, and impairing lipid degradation/export which may cause cellular lipotoxicity[152]. Approximately 40%-80% of CHC individuals have steatohepatitis due to viral pathogenesis, which is associated with the increased risk of HCC[153]. The HCV core protein is a key player in altering lipid metabolism through (Figure 5): (1) Decreasing lipid turnover of HCV core particles coated lipid droplets (LD); (2) Inhibition of LD mobility; (3) Inhibition of microsomal triglyceride transfer protein which prevents lipid export and degradation; and (4) Inhibition of peroxisome proliferator-activating receptor-α/γ; inhibition of diacylglycerol acetyltransferase 1[154,155]. Accumulation of free fatty acids causes severe mitochondrial and ER oxidative stress. The accumulation or ROS stimulates lipid peroxidation and activation of inflammatory signaling cascades such as TNF-α and IL-1 which can lead to the development of steatohepatitis and insulin resistance[156].

Deregulation of cellular pathways by HCV proteins

Activation of cell-survival and growth pathways are mediated by core, E2, NS2, NS3, NS4A NS5a, and NS5B proteins (Figure 5). To promote cell cycling and evasion of the G1/S checkpoint, NS5B binds to tumor suppressor Rb to facilitate proteasomal degradation and release of E2F to produce cell-cycle dependent genes[157]. NS2 activates the cyclin D/CDK 4 complex to induce the expression of cyclin E[158]. The core protein upregulates cyclin E/CDK 2 to promote cell cycle transition from G1 to S phase with checkpoint evasion, genome instability, and aberrant cell growth[159]. NS5A inactivates the tumor suppressor pTEN through binding, to cause proliferative growth and survival using the PI3K/Akt pathway[160]. HCV Core, E2, NS3, and NS5A interact with various proteins in the RAF/MAPK/ERK pathways to promote cellular proliferation[161-164]. The Wnt/β-catenin signaling pathway is activated by direct binding of β-catenin by NS5A or phospho-inactivation of GSK-3β by NS5A and core proteins[165-168]. Activation of Wnt target genes promotes proliferation, angiogenesis and EMT transfer. High quantities of β-catenin are associated with poor prognosis of HCC[169].

The inhibition of apoptosis contributes to the development of HCC through the growth of abnormal cells. The tumor suppressor protein, p53, is targeted by many HCV proteins to prevent apoptosis, DNA-repair, and senescence. NS5A directly binds to p53 causing inhibition, while NS2, NS3/4A interfere with the p53 pathway by inducing p53 delocalization from the nucleus to the cytoplasm or perinuclear regions[170-173]. There is some evidence that the HCV core protein is also able to bind to p53, however, this is debated, because high levels of core cause repression while low levels promote p53 activity[174]. To avoid cell death, HCV also has various protein mechanisms to inhibit TNF-α cytokine-mediated apoptosis: (1) The core protein activates FLICE, an inhibitor of TNF-α signaling[175]; (2) NS5A protein prevents TNF-α-mediated cell death by inhibiting activation of caspase-3 and PARP1 cleavage[176]; and (3) NS5A can also interact with intrinsic apoptosis regulator Bid to inhibit activation of apoptosis[177].

The expression of TGF- β signaling is antiproliferative and pro-apoptosis[178]. HCV NS5A binds directly to the TGF- β receptor 1 to block signaling, and as such prevents phosphorylation and nuclear localization of smad2 and the smad3/smad4 heterodimer[179]. Mutant core proteins derived from HCV tumors inhibit the TGF- β pathway through direct interaction with Smad3, which results in the inhibition of DNA-binding by the Smad3/4 heterodimer[180]. Inhibition of the TGF- β pathway promotes EMT which enhances fibrogenesis, tumor invasion, and metastasis[178].

COMMON SOMATIC MUTATIONS IN PROGRESSIVE HCC TUMORS

As the progression of liver disease to HCC occurs, many common driver mutations that allow for selective growth advantage for tumor cells over normal cells can be identified (Figure 7). HCC tumors are highly heterogeneous within the same individual and these differences in tumor genetic profiles are amplified by single cell and next-generation sequencing. From sequence analysis several driver genes have been identified in the progression of HCC: TERT, tumor protein p53 (TP53), catenin beta 1 (CTNNB1), Wnt/β-catenin signaling protein AXIN1, chromatin remodeling genes ARID1A and ARID2, oxidative stress response genes NFE2L2 and KEAP1, RAS/MAPK signaling (RPS6KA3), and the JAK/STAT signaling cascade activator (KAK1). The most disrupted driver genes are described below, for a comprehensive overview refer to[181].

Regardless of geographic location, recurrent somatic mutations in the TERT promoter have been identified as the most common mutation in HCC (20.7%-59%)[181]. The TERT protein has an important role in maintaining telomere length by adding short-repeated TTAGGG nucleotides at the end of chromosomes[182]. Maintenance of the telomeres is important to avoid DNA damage, however normal adult cells do not express TERT and can only undergo 40-60 cycles of replication before senescence[183]. In HCC, activating mutations in the TERT promoter enable replicative immortality through the consistent addition of telomeric repeats which allow cells expressing TERT to replicate without entering senescence[182,183]. TERT mutations have been identified to occur early in malignant transformation and persist throughout tumor progression[184]

The tumor suppressor protein P53 is a critical protein commonly mutated in cancer, that is involved in cell-cycle arrest at the G1/S checkpoint and activation of apoptosis[185]. TP53 is most frequently mutated in its DNA binding domain, to prevent

its role in activating TP53 responsive genes that aid in cell-cycle control[186]. Loss of p53 function aids cell transformation through constant cell cycling without DNA damage checkpoint regulation[5]. Mutational frequency of P53 in HCC is dependent on geographic locations, as mutational frequency requires aflatoxin exposure. High dietary aflatoxin exposure and endemic hepatitis B infection is associated with TP53 mutations located on R249S/V157F and poor prognosis[187,188].

High mutational frequency in the CTNNB1 gene which codes for the β-catenin transcription factor in the Wnt-pathway is associated with tumor progression and poor prognosis[5]. Activating mutations in CTNNB1 increase cytoplasmic accumulation of β-catenin without a Wnt signaling molecule[189]. Normally without a signaling molecule, β-catenin is expressed, bound by the destruction complex, phosphorylated by GSK3, and degraded by the proteasome[189]. However, in the presence of a Wnt ligand signal, β-catenin is not degraded and instead it is expressed to high levels and translocates into the nucleus and activates transcription factor TCF to transcribe Wnt target genes[190]. These target genes are involved in growth, proliferation, and EMT promoting metastasis[5,191]. In HCC, the tumor suppressor AXIN1 is the second most mutated gene (6.8%) in the Wnt-signaling pathway. AXIN1 is involved as a scaffold protein for the β-catenin destruction complex, and inactivating mutations prevent the complex from forming and destroying β-catenin, thus leading to increased β-catenin levels leading to tumor growth and proliferation[190,192]. The deregulation of Wnt/β-catenin signaling has been observed in 40%-70% of HCC patients. Moreover, β-catenin associated mutations occur in lower frequencies in HBV-related HCC, and are more common in HCV and alcohol-related HCC[181].

The ARID1A and ARID2 genes encode for a key subunit component of the SWI/SNF chromatin remodeling complex[193]. The dysregulation of the SWI/SNF complex contributes to tumor heterogeneity, and drug treatment resistance[181]. ARID1A acts as a tumor suppressor gene that is expressed at high levels in normal livers to regulate DNA activity using nucleosomes to restrict cell proliferation[5]. Inactivating mutations in the ARID1A and ARID2 genes occur in around 10% of HCC cases and are associated with poor prognosis, increased cell proliferation, and migration/metastases of HCC cells[181,193,194].

CONSIDERATIONS FOR THE FUTURE

The low 5-year survival rate for those diagnosed with HCC is largely due to the failure of early detection of small lesions and lack of medical therapy for advanced disease[195]. The best curative treatment option for HCC is liver transplantation, however, this is limited to those who are in the early stages of HCC and follow the Milan transplantation criteria or the Alberta HCC algorithm in Canada[196,197]. Other available treatment options during earlier stages of HCC include surgical resection (70% 5-year survival rate) and radiofrequency ablation therapy (40%-70% 5-year survival rate in tumors < 2 cm)[198,199]. During intermediate HCC disease stages, transarterial chemoembolization treatment can be offered (median survival rate 16-20 mo)[200]. In advanced disease, HCC is often quite unresponsive to most chemotherapeutics, and current chemotherapeutics (Sorafenib and Lenvatinib) that target overexpressed receptor-tyrosine-kinase pathways (*e.g.* vascular endothelial growth factor, MAPK, EGFR, RAS, IGF, PI3K/PTEN/Akt/mTOR, Wnt/beta-catenin) only increase median survival by three months[201]. In 2018, the monoclonal antibody nivolumab which targets the programed cell death 1 receptor on T-cells was approved as a second-line therapeutic for HCC. Nivolumab has a 20% response rate in initial phase II clinical trials and works by activating T-cells for the immune-mediated killing of tumors[196].

Exploiting traits of virally induced cancers as therapeutics

An important feat for the future of HCC treatment will be the development of effective immunotherapies. Nivolumab was the first monoclonal antibody approved for advanced-stage HCC treatment and there are many others that are currently in clinical trials. Since both chronic viral infection and cancer create an immunosuppressive environment to prevent cytotoxic killing, future immunotherapies could target regulatory (T_{reg}) and resident memory T-cells (T_{RM}) to reactivate the immune system against viral infection and HCC tumors[202]. Additionally, molecular pathways deregulated by HBV/HCV/HDV could be targeted by immunotherapies through inhibition of pathways involved in aberrant cell growth leading to HCC. Alternatively, tumors caused by viral etiologies can be targeted through training of the immune

system to target viral particles and/or fusion proteins in HCC[203]. Tan *et al*[203], describes a CAR-T cell technology that can recognize HBV specific epitopes in HCC tumors. Since HBV-associated tumors do not contain actively replicating viruses and only express partially integrated/truncated proteins, T-cells can be designed to target these tumors associated antigens. One of the patients from this study treated with HBV specific CAR-T cells had a decreased tumor volume in 5 of 6 pulmonary metastases over the course of 1-year. Building on the study performed by Tan *et al*[203], another possibility to improve the persistence of CAR-T technology in viral related HCC could be through engineering a separate CAR-T receptor to recognize viral antigens to boost T-cell populations while targeting cancer-specific lesions. Although immunotherapeutics for solid tumors is in its infancy, this is likely the future for the development of better treatment options for HCC.

CONCLUSION

Chronic hepatitis B, C, and delta viral infections affect almost half a billion people worldwide. Decades-long persistent viral infection and immune-mediated damage cause significant changes in the liver microenvironment and are the strongest risk factors for the development of HCC. Current treatment options for HBV and HCV reduce HCC risk, but do not eliminate it. Moreover, the lack of an HBV virological cure and limited treatment options for HDV requires the exploration of more effective treatments.

HBV and HCV can manipulate pathways in ten hallmarks of cancer, which may explain how these viruses escalate risk of HCC development. HDV is not considered a "directly" oncogenic virus, due to HDV reliance on HBV to complete the viral life cycle, uses several mechanisms to aid the progression of liver disease and increase the risk of HCC[46]. Successful epidemiology studies, *in-vitro* cell culture studies, and animal studies have provided us with significant insight into the molecular mechanisms of interactions between host-viral interactions. Genomic analyses comparing HCC tumors to those of healthy tissue have provided us insight into driver mutations that aid in the progression of viral-mediated HCC and possible targets for future treatments. Although much progress has been made in the field, there is a lot that remains unknown due to the lack of cell-culture systems that can be used to study all viral genotypes, co-infections, and animal models that can be infected with these viruses to produce liver disease similar to humans. The development of stronger experimental models will provide us with further insight into the role these viruses play in promoting HCC development. Until we have better screening methods and more accessible and/or effective antiviral treatments, the rates of liver cancer will be steadily on the rise. Thus, we need to investigate commonly deregulated pathways in HCC to identify targets and develop more effective treatments to improve the survival rate for those diagnosed with this disease.

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MINIREVIEWS

Role of artificial intelligence in the diagnosis of oesophageal neoplasia: 2020 an endoscopic odyssey

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Abstract

The past decade has seen significant advances in endoscopic imaging and optical enhancements to aid early diagnosis. There is still a treatment gap due to the underdiagnosis of lesions of the oesophagus. Computer aided diagnosis may play an important role in the coming years in providing an adjunct to endoscopists in the early detection and diagnosis of early oesophageal cancers, therefore curative endoscopic therapy can be offered. Research in this area of artificial intelligence is expanding and the future looks promising. In this review article we will review current advances in artificial intelligence in the oesophagus and future directions for development.

Key Words: Artificial intelligence; Oesophageal neoplasia; Barrett's oesophagus; Squamous dysplasia; Computer aided diagnosis; Deep learning

Core Tip: Computer aided diagnosis of oesophageal pathology may potentially be an adjunct for the endoscopist which will improve the detection of early neoplasia in Barrett's oesophagus and early squamous neoplasia such that curative endoscopic therapy can be

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offered. There are significant miss rates of oesophageal cancers despite advances in endoscopic imaging modalities and an artificial intelligence (AI) tool will off-set human factors associated with some miss rates. To fulfil the potential of this exciting area of AI certain criteria need to be met which we will expand upon. Once implemented this will have a significant impact on this field of endoscopy.

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INTRODUCTION

The past decade has seen significant advances in endoscopic imaging and optical enhancements to aid early diagnosis. Oesophageal cancer (adenocarcinoma and squamous cell carcinoma) is associated with significant mortality[1]. As of 2018 oesophageal cancer was ranked seventh in the world in terms of cancer incidence and mortality, with 572000 new cases[2]. Oesophageal squamous cell carcinoma accounts for more than 90% of oesophageal cancers in china with an overall 5-year survival rate less than 20%[3].

Despite the technological advances there is still a treatment gap due to the underdiagnosis of lesions of the oesophagus[4]. A metanalysis of 24 studies showed that missed oesophageal cancers are found within a year of index endoscopy in a quarter of patients undergoing surveillance for Barrett's oesophagus (BE)[5]. A large multicentre retrospective study of 123395 upper gastrointestinal (GI) endoscopies showed an overall missed oesophageal cancer rate of 6.4%. The interval between a negative endoscopy and the diagnosis was less than 2 years in most cases[6]. Multivariate analysis showed that one of the factors associated with the miss rate is a less experienced endoscopist.

Efforts are necessary to improve the detection of early neoplasia secondary to BE and early squamous cell neoplasia (ESCN) such that curative minimally invasive endoscopic therapy can be offered to patients. Computer aided diagnosis may play an important role in the coming years in providing an adjunct to endoscopists in the early detection and diagnosis of early oesophageal cancers.

In this review article we will review current advances in artificial intelligence in the oesophagus and future directions for development.

DEFINITIONS

Machine learning is the use of mathematical models to capture structure in data[7]. The algorithms improve automatically through experience and do not need to be explicitly programmed[8]. The final trained models can be used to make prediction of oesophageal diagnosis. Machine learning is classified into supervised and unsupervised learning. During supervised learning, the model is trained with data containing pairs of inputs and outputs. It learns how to map the inputs and outputs and applies this to unseen data. In unsupervised learning the algorithm is given data inputs which are not directly linked to the outputs and therefore has to formulate its own structure and set of patterns from the inputs[9].

Deep learning is a subtype of machine learning in which the model, a neural network, is composed of several layers of neurons, similar to the human brain. This enables automatic learning of features, which is particularly useful in endoscopy where images and videos lack structure and are not easily processed into specific features[9]. A convolutional neural network (CNN) is a subtype of deep learning which can take an input endoscopic image and learn specific features (*e.g.*, colour, size, pit pattern), process the complex information through many different layers and produce an output prediction (*e.g.*, oesophageal dysplasia or no dysplasia) (Figure 1).

To develop a machine learning model, data needs to be split into 3 independent groups-training set, validation set and testing set. The training set is used to build a model using the oesophageal labels (*e.g.*, dysplasia or no dysplasia). The validation set

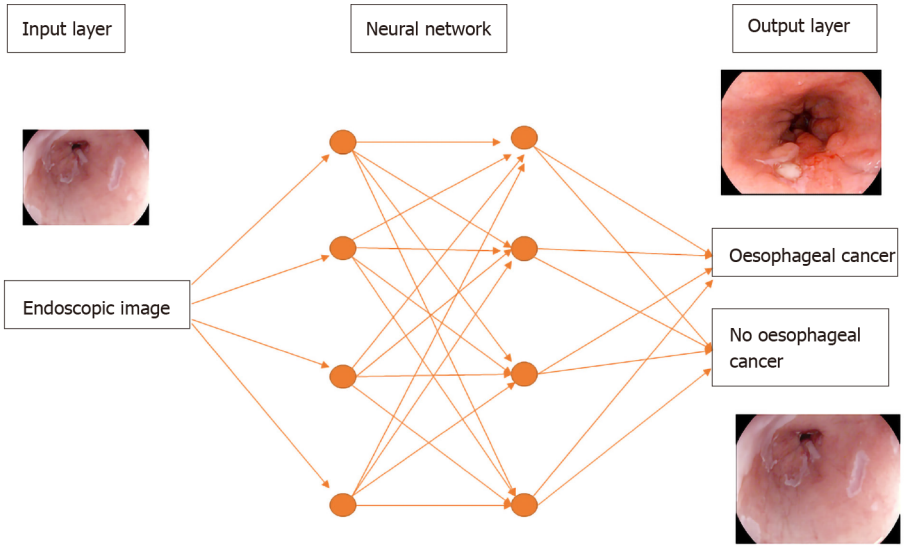

Figure 1 A deep learning model. Features of an endoscopic image processed through multiple neural layers to produce a predicted diagnosis of oesophageal cancer or no oesophageal cancer present on the image.

provides an unbiased evaluation of the model's skill whilst tuning the hyper-parameters of the model, for example, the number of layers in the neural network. It is used to ensure that the model is not overfitting to the training data. Overfitting means that the model will perform well on the training data but not on the unseen testing data. The test set is used to evaluate the performance of the predictive final model[7] (Figure 2).

ADVANCES IN ENDOSCOPIC IMAGING

Endoscopic imaging has advanced into a new era with the development of high definition digital technology. A charge coupled device chip in standard white light endoscopy produces an image signal of 10000 to 400000 pixels displayed in a standard definition format. The chips in a high definition white light endoscope produce image signals of 850000 to 1.3 million pixels displayed in high definition[10]. This has improved our ability to pick up the most subtle oesophageal mucosal abnormalities by assessing mucosal pit patterns and vascularity to allow a timely diagnosis of dysplasia or early cancer.

There have been further advances in optical technology in the endoscope with chromoendoscopy such as narrow-band imaging (NBI), i-scan (Pentax, Hoya) and blue laser imaging (Fujinon), which have further improved early neoplasia detection and diagnosis in the oesophagus. Table 1 summarises some of the studies investigating the accuracy of these imaging modalities in detecting BE dysplasia by formulating classification systems based on mucosal pit pattern, colour and vascular architecture.

In squamous epithelium the microvascular vascular patterns of intrapapillary capillary loops (IPCL) is used to aid in the diagnosis of early squamous cell cancer (Figure 3)[14]. The classification systems that are currently used are based on magnification endoscopy assessment of IPCL patterns[15].

The disordered and distorted mucosal and vascular patterns used to define the above classifications can be used to train the CNN to detect early cancer in the oesophagus.

BE AND EARLY CANCER

BE is the only identifiable premalignant condition associated with invasive oesophageal adenocarcinoma. There is a linear progression from non-dysplastic BE, to low grade and high-grade dysplasia. Early neoplasia which is confined to the mucosa have significant eradication rates of > 80%[16].

The standard of care for endoscopic surveillance for patients with BE are random biopsies taken as part of the Seattle protocol where four-quadrant biopsies are taken

Table 1 Studies showing accuracy in the detection of Barrett's oesophagus dysplasia for each endoscopic modality

	I-scan optical enhancement	NBI	BLI
Ref.	Everson *et al*[11]	Sharma *et al*[12]	Subramaniam *et al*[13]
Features assessed	Mucosal pit pattern, vessels	Mucosal pit pattern, vessels	Colour, mucosal pit patterns, vessels
Accuracy	Experts = 84%, non-experts = 76%	85%	Experts = 95.2%, non-experts = 88.3%
Sensitivity	Experts = 77%, non-experts = 81%	80%	Experts = 96%, non-experts = 95.7%
Specificity	Experts = 92%, non-experts = 70%	88%	Experts = 94.4%, non-experts = 80.8%

NBI: Narrow-band imaging; BLI: Blue laser imaging.

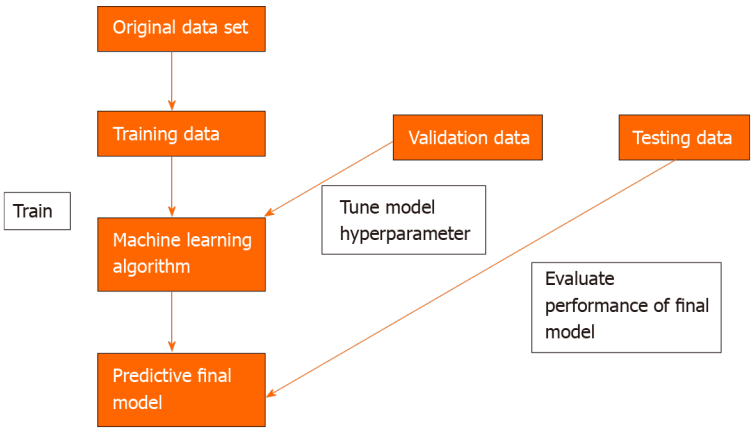

Figure 2 Three independent data sets are required to create a machine learning model that can predict an oesophageal cancer diagnosis.

every 2 cm of BE[17]. This method is not perfect and is associated with sampling error. The area of a 2 cm segment of BE is approximately 14 cm^2, a single biopsy sample is approximately 0.125 cm^2. Therefore, Seattle protocol biopsies will only cover 0.5 cm^2 of the oesophageal mucosa which is 3.5% of the BE segment[18]. Dysplasia can often be focal and therefore easily missed. Studies have also shown that compliance with this protocol is poor and is worse on longer segments of BE[19].

The American Society for Gastrointestinal Endoscopy preservation and incorporation of valuable endoscopic innovations (PIVI) initiative was developed to direct endoscopic technology development. Any imaging technology with targeted biopsies in BE would need to achieve a threshold per patient sensitivity of at least 90% for the detection of high-grade dysplasia and intramucosal cancer. It would require a specificity of at least 80% in BE in order to eliminate the requirement for random mucosal biopsies during BE endoscopic surveillance. This would improve the cost and effectiveness of a surveillance programme. This is the minimum target an AI technology would need to meet in order to be able to be ready for prime time and a possible adjunct during a BE surveillance endoscopy[20].

An early study tested a computer algorithm developed based on 100 images from 44 patients with BE. It was trained using colour and texture filters. The algorithm diagnosed neoplastic lesions on a per image level with a sensitivity and specificity of 0.83. At the patient level a sensitivity and specificity of 0.86 and 0.87 was achieved respectively. This was the first study where a detection algorithm was developed for detecting BE lesions and compared with expert annotations[21].

A recent study developed a hybrid ResNet-UNet model computer aided diagnosis system which classified images as containing neoplastic or non-dysplastic BE with a sensitivity and specificity of 90% and 88% respectively. It achieved higher accuracy than non-expert endoscopists[22].

De Groof *et al*[23] performed one of the first studies to assess the accuracy of a computer-aided detection (CAD) system during live endoscopic procedures of 10 patients with BE Dysplasia and 10 patients without BE dysplasia. Three images were evaluated every 2 cm of BE by the CAD system. Sensitivity and specificity of the CAD system in per level analysis was 91% and 89% respectively (Figure 4).

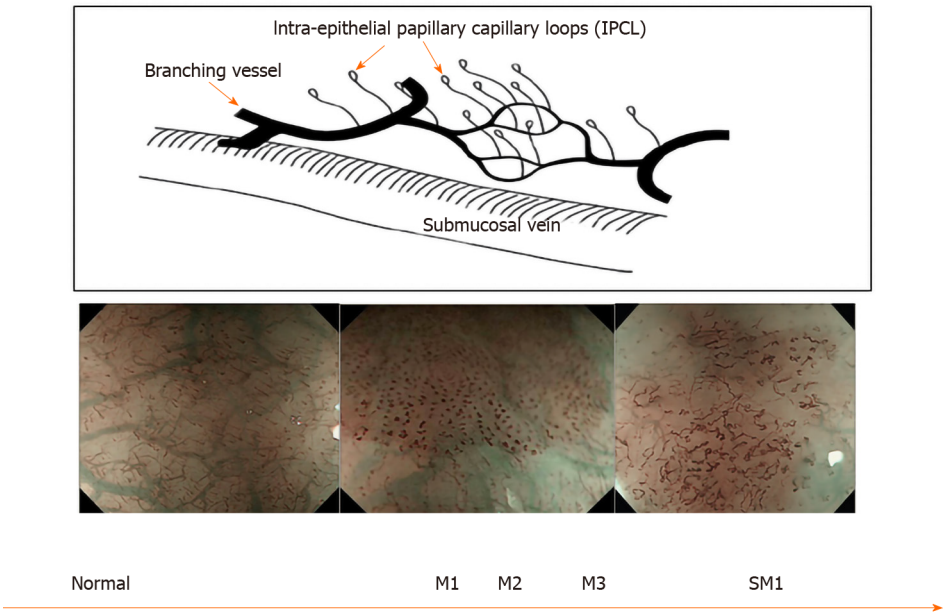

Figure 3 Intrapapillary capillary loops patterns during magnification endoscopy to assess for early squamous cell neoplasia and depth of invasion. M1, M2, M3 = invasion of epithelium, lamina propria and muscularis propria respectively. SM1= superficial submucosal invasion. Citation: Inoue H, Kaga M, Ikeda H, Sato C, Sato H, Minami H, Santi EG, Hayee B, Eleftheriadis N. Magnification endoscopy in esophageal squamous cell carcinoma: a review of the intrapapillary capillary loop classification. *Ann Gastroenterol* 2015; 28: 41-48. Copyright© The Authors 2015. Published by Hellenic Society of Gastroenterology.

Hussein *et al*[24] developed a CNN trained using a balanced data set of 73266 frames from BE videos of 39 patients. On an independent validation set of 189436 frames from 19 patients the CNN could detect dysplasia with a sensitivity of 88.3% and specificity of 80%. The annotations were created from and tested on frames from whole videos minimising selection bias.

Volumetric laser endomicroscopy (VLE) is a wide field imaging technology used to aid endoscopists in the detection of dysplasia in BE. An infrared light produces a circumferential scan of 6cm segments of BE up to a depth of 3 mm allowing the oesophageal layer and submucosal layer with its associated vascular networks to be visualized[25]. The issue is there is large volumes of complex data which the endoscopist needs to interpret. An Artificial intelligence system called intelligent real-time image segmentation has been used to interpret the data produced from VLE. This software identifies 3 VLE features associated with histological evidence of dysplasia and displays the output with colour schemes. A hyper reflective surface (pink colour) suggests that there is increased surface maturation, cellular crowding and increased nuclear-to-cytoplasmic ratio. Hyporeflective structures (blue colour) suggests abnormal morphology of BE epithelial glands. A lack of layered architecture (orange colour) differentiates squamous epithelium from BE (Figure 5)[26]. A recent study analysed ex-vivo images from 29 BE patients with and without early cancer retrospectively. A CAD system which analysed multiple neighbouring VLE frames showed improved neoplasia detection in BE relative to single frame analysis with an AUC of 0.91[27].

Table 2 provides a summary of all the studies investigating the development of deep learning algorithms for the diagnosis of early neoplasia in BE.

ESCN

With advances in endoscopic therapy in recent years ESCN confined to the mucosal layer can be curatively resected endoscopically with a < 2% incidence of local lymph node metastasis. IPCL are the microvascular features which can be endoscopically used to help classify and identify ESCN and if there is a degree of invasion in the muscularis mucosa and submucosal tissue[16].

Lugols chromoendoscopy is a screening method for identifying ESCN during an upper GI endoscopy. However, despite a sensitivity of > 90%, it is associated with a low specificity of approximately 70%[32]. There is also a risk of allergic reaction with

Table 2 Summary of all the studies investigating the development of machine learning algorithms for the detection of dysplasia in Barrett's oesophagus

Ref.	Year	Endoscopic processor	Study design	Study aim	Algorithm used	No. of patients	No. of BE images	Sensitivity	Specificity
Van der Sommen *et al*[21]	2016	WLE Fujinon	Retrospective	Assess feasibility of computer system to detect early neoplasia in BE	Machine learning, specific textures and colour filters	44	100 (60 dysplasia, 40 NDBE)	83% (per image), 86% (per patient)	83% (per image), 87% (per patient)
Sweger *et al*[28]	2017	VLE	Retrospective	Assess feasibility of computer algorithm to identify BE dysplasia on *ex vivo* VLE images	Several machine learning methods; discriminant analysis, support vector machine, AdaBoost, random forest, K-nearest neighbors	19	60 (30 dysplasia, 30 NDBE)	90%	93%
Ebigbo *et al*[29]	2018	WLE, NBI, Olympus	Retrospective	Detection of early oesophageal cancer	Deep CNN with a residual net architecture	50 with early neoplasia	248	97% (WLE), 94% (NBI)	88% (WLE), 80% (NBI)
de Groof *et al*[30]	2019	WLE, Fujinon	Prospective	Develop CAD to detect early neoplasia in BE	Supervised Machine learning. Trained on colour and texture features	60	60 (40 dysplasia, 20 NDBE)	95%	85%
de Groof *et al*[22]	2020	WLE Fujinon, WLE Olympus	Retrospective, Prospective	Develop and validate deep learning CAD to improve detection of early neoplasia in BE	CNN pretrained on GastroNet. Hybrid ResNet/U-Net model	669	1704 (899 dysplasia, 805 NDBE)	90%	88%
Hashimoto *et al*[31]	2020	WLE, Olympus	Retrospective	Assess if CNN can aid in detecting early neoplasia in BE	CNN pretrained on image net and based on Xception architecture and YOLO v2	100	1832 (916 dysplasia, 916 NDBE)	96.4%	94.2%
de Groof *et al*[23]	2020	WLE, Fujinon	Prospective	Evaluate CAD assessment of early neoplasia during live endoscopy	CNN pretrained on GastroNet; hybrid ResNet/U-Net Model	20	-	91%	89%
Struyvenberg MR *et al*[27]	2020	VLE	Prospective	Evaluate feasibility of automatic data extraction followed by CAD using mutiframe approach to detect to dysplasia in BE	CAD multiframe analysis with principal component analysis	29	-	-	-

BE: Barrett's oesophagus; WLE: White light endoscopy; NBI: Narrow band imaging; VLE: Volumetric laser endomicroscopy; CNN: Convolutional neural network; CAD: Computer-aided detection.

iodine staining. Advanced endoscopic imaging with NBI has a high accuracy for detecting ESCN however a randomised control trial showed its specificity was approximately 50%[33]. Computer assisted detection systems have been developed to try and overcome many of these issues which aid endoscopists in detecting early ESCN lesions.

Everson *et al*[16] developed a CNN trained with 7046 sequential high definition magnification endoscopy with NBI. These were classified by experts using the IPCL patterns and based on the Japanese Endoscopic Society classification. The CNN was able to accurately classify abnormal IPCL patterns with a sensitivity and specificity of 89% and 98% respectively. The diagnostic prediction times were between 26 and 37 ms (Figure 6).

Nakagawa *et al*[34] developed a deep learning-based AI algorithm using over 14000 magnified and non-magnified endoscopic images from 804 patients. This was able to

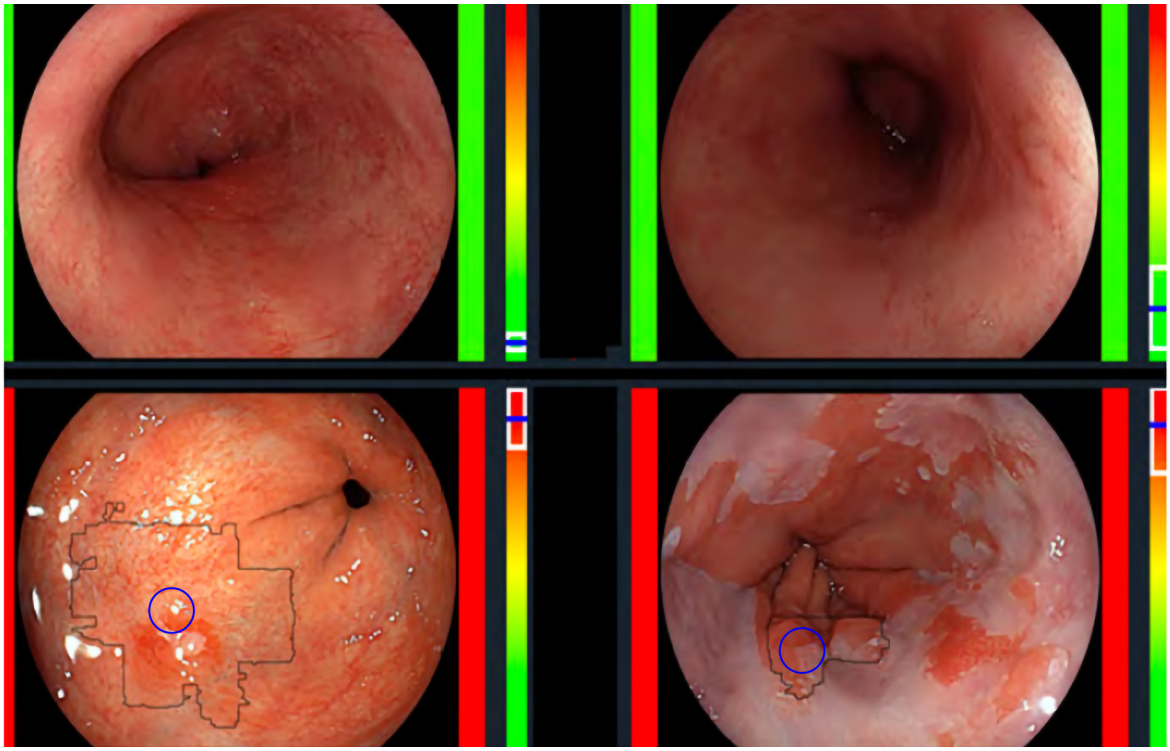

Figure 4 The computer-aided detection system providing real time feedback regarding absence of dysplasia (top row) or presence of dysplasia (bottom row). Citation: de Groof AJ, Struyvenberg MR, Fockens KN, van der Putten J, van der Sommen F, Boers TG, Zinger S, Bisschops R, de With PH, Pouw RE, Curvers WL, Schoon EJ, Bergman JJGHM. Deep learning algorithm detection of Barrett's neoplasia with high accuracy during live endoscopic procedures: a pilot study (with video). *Gastrointest Endosc* 2020; 91: 1242-1250. Copyright© The Authors 2020. Published by Elsevier.

predict the depth of invasion of ESCN with a sensitivity of 90.1% and specificity of 95.8% (Figure 7).

Guo *et al*[3] trained a CAD system using 6473 NBI images for real time automated diagnosis of ESCN. The deep learning model was able to detect early ESCN on still NBI images with a sensitivity of 98% and specificity of 95%. On analysis of videos the per frame sensitivity was 60.8% on non-magnified images and 96.1% on magnified images. The per lesion sensitivity was 100%. This model had high sensitivity and specificity in both still images and real time video setting the scene for the development of better models for real time detection of early ESCN.

Endocytoscopy uses a high-power fixed-focus objective lens attached to the endoscope to give ultra-high magnification images. The area of interest is stained to allow identification of cellular structures like in standard histopathology techniques. This allows the endoscopist to characterise ESCN and make a real time histological diagnosis[35].

Kumagai *et al*[36] developed a CNN trained using more than 4000 endocytoscopic images of the oesophagus (malignant and non-malignant). The AI was able to diagnose esophageal squamous cell carcinoma with a sensitivity of 92.6%. This provides a potential AI tool which can aid the endoscopist by making an *in vivo* histological diagnosis. This would allow endoscopists to make a clinical decision in the same endoscopic session regarding resection of the early oesophageal cancer which would potentially save on costs by replacing the need for protocol biopsies

Table 3 provides a summary of all the studies investigating the development of deep learning algorithms for the diagnosis of ESCN.

AI AND HISTOLOGY ANALYSIS IN OESOPHAGEAL CANCER

In digital pathology tissue slides are scanned as high-resolution images as each slide contains a large volume of cells. The cellular structure needs be visible to the histopathologist in order to identify areas of abnormality[43]. Histopathological analysis often requires a lot of time, high costs and often manual annotation of areas of interest by the histopathologists. There is also a possible miss rate of areas of early oesophageal dysplasia as the area can be focal. There is also suboptimal interobserver agreement

Table 3 Summary of all the studies investigating the development of machine learning algorithms for the detection of early squamous cell neoplasia

Ref.	Year	Endoscopic processor	Study design	Study aim	Algorithm used	No. of patients	No. of images	Sensitivity	Specificity
Shin *et al*[37]	2015	High resolution micro-endoscopy	Retrospective	Differentiate neoplastic and non-neoplastic squamous oesophageal mucosa	Quantitative image analysis. Two-class linear discriminant analysis to develop classifier	177	375	87%	97%
Quang *et al*[38]	2016	High resolution micro-endoscopy	Retrospective	Differentiate neoplastic and non-neoplastic squamous oesophageal mucosa	Two-class linear discriminant analysis to develop classifier	3	-	95%	91%
Horie *et al*[39]	2018	WLE, NBI, Olympus	Retrospective	Ability of AI to detect oesophageal cancer	Deep CNN (Multibox detector architecture)	481	-	97%	-
Everson *et al*[16]	2019	Magnified NBI, Olympus	Retrospective	Develop AI system to classify IPCL patterns as normal/abnormal in endoscopically resectable lesions real time	CNN, explicit class activation maps generated to depict area of interest for CNN	17	7046	89%	98%
Nakagawa *et al*[34]	2019	Magnified and non-magnified, NBI, BLI, Olympus, Fujifilm	Retrospective	Predict depth of invasion of ESCN	Deep CNN (multibox detector architecture)	959	15,252	90.1%	95.8%
Kumagai *et al*[36]	2019	ECS	Retrospective	Deep learning AI to analyse ECS images as possible replacement of biopsy-based histology	CNN constructed based on GoogLeNet	-	6235	92.6%	89.3%
Zhao *et al*[40]	2019	ME NBI, Olympus	Retrospective	Classification of IPCLs to improve ESCN detection	A double-labeling fully convolutional network	219	-	87%	84.1%
Guo *et al*[3]	2020	ME and non-ME NBI, olympus	Retrospective	Develop a CAD for real-time diagnosis of ESCN	Model based on SegNet architecture	2672	13144 images (4250 malignant, 8894 non-cancerous), 168865 video frames	Images = 98.04%, non-magnified video = 60.8%, magnified video = 96.1%	Images = 95.03%, non-magnified /magnified video = 99.9%
Tokai *et al*[41]	2020	WLE, NBI, Olympus	Retrospective	Ability of AI to measure squamous cell cancer depth	Deep CNN	-	2044	84.1%	73.3%
Ohmori *et al*[42]	2020	Magnified and non-magnified, WLE, NBI, BLI, Olympus, Fujifilm	Retrospective	Detect Oesophageal squamous cell cancer	CNN	-	11806 non- magnified images, 11483 magnified images	Non-ME WLE = 90%, non-ME NBI/BLI = 100%, ME = 98%	Non-ME WLE = 76%, non-ME NBI/BLI = 63%, ME = 56%

WLE: White light endoscopy; NBI: Narrow band imaging; AI: Artificial intelligence; CNN: Convolutional neural network; IPCL: Intrapapillary capillary loops; BLI: Blue laser imaging; ESCN: Early squamous cell neoplasia; ECS: Endocytoscopic system; ME: Magnification endoscopy.

among expert GI histopathologists in certain histological diagnosis such as low-grade dysplasia in BE[44].

A novel AI system to detect and delineate areas of early oesophageal cancer on histology slides could be a key adjunct to histopathologists and help improve

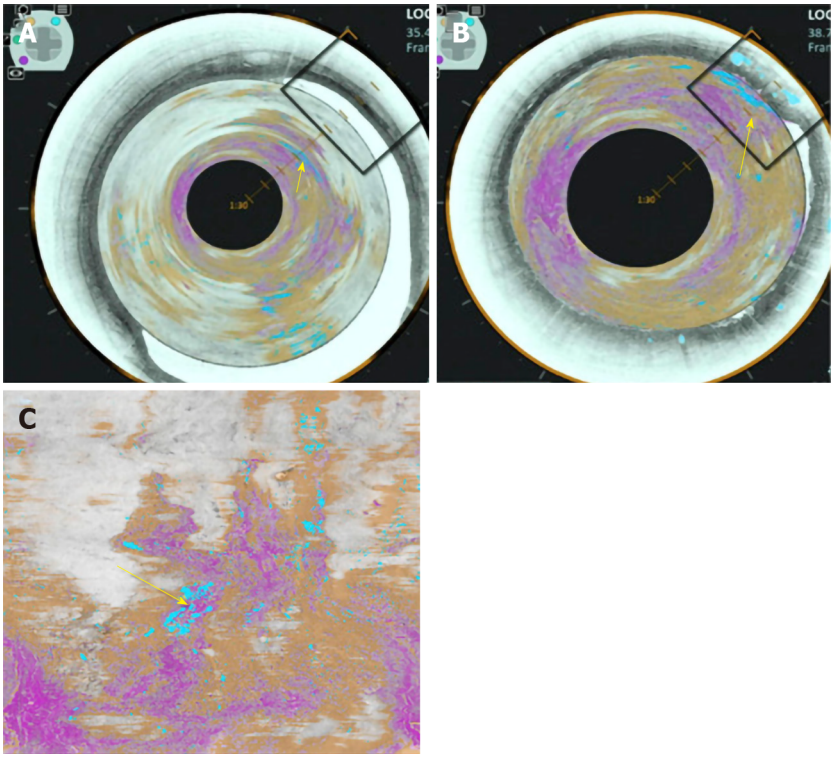

Figure 5 Volumetric laser endomicroscopy image showing area of overlap (yellow arrow) between the 3 features of dysplasia identified with the colour schemes. A: View looking down into the oesophagus; B: Close up of dysplastic area; C: Forward view of the dysplastic area. A-C: Citation: Trindade AJ, McKinley MJ, Fan C, Leggett CL, Kahn A, Pleskow DK. Endoscopic Surveillance of Barrett's Esophagus Using Volumetric Laser Endomicroscopy With Artificial Intelligence Image Enhancement. *Gastroenterology* 2019; 157: 303-305. Copyright© The Authors 2019. Published by Elsevier.

detection and delineation of early oesophageal cancer.

Tomita *et al*[43] developed a convolutional attention-based mechanism to classify microscopic images into normal oesophageal tissue, BE with no dysplasia, BE with dysplasia and oesophageal adenocarcinoma using 123 histological images. Classification accuracy of the model was 0.85 in the BE-no dysplasia group, 0.89 in the BE with dysplasia group, and 0.88 in the oesophageal adenocarcinoma group.

ROLE OF AI IN QUALITY CONTROL IN THE OESOPHAGUS

The inspection time of the oesophagus and clear mucosal views have an impact on the quality of an oesophagoscopy and the yield of early oesophageal neoplasia detection. Assessment should take place with the oesophagus partially insufflated between peristaltic waves. An overly insufflated oesophagus can flatten a lesion which can in turn be missed[45]. The British Society of Gastroenterology consensus guidelines on the Quality of upper GI endoscopy recommends adequate mucosal visualisation achieved by a combination of aspiration, adequate air insufflation and use of mucosal cleansing techniques. They recommend that the quality of mucosal visualisation and the inspection time during a Barrett's surveillance endoscopy should be reported[46].

Chen *et al*[47] investigated their AI system, ENDOANGEL, which provides prompting of blind spots during upper GI endoscopy, informs the endoscopist of the inspection time and gives a grading score of the percentage of the mucosa that is visualised.

CONCLUSION

Computer aided diagnosis of oesophageal pathology may potentially be a key adjunct for the endoscopist which will improve the detection of early neoplasia in BE and ESCN such that curative endoscopic therapy can be offered. There are significant miss rates of oesophageal cancers despite advances in endoscopic imaging modalities and an AI tool will off-set the human factors associated with some of these miss rates.

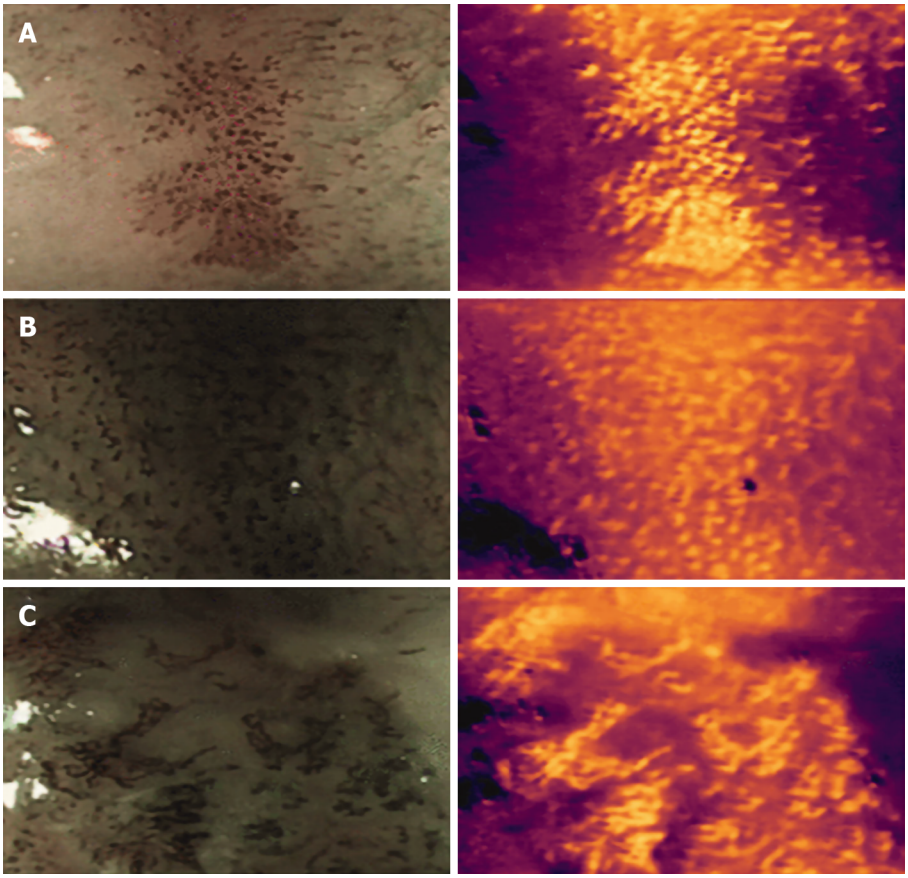

Figure 6 Input images on the left and corresponding heat maps on the right illustrating the features recognised by the convolutional neural network when classifying images by recognising the abnormal intrapapillary capillary loops patterns in early squamous cell neoplasia. Citation: Everson M, Herrera L, Li W, Luengo IM, Ahmad O, Banks M, Magee C, Alzoubaidi D, Hsu HM, Graham D, Vercauteren T, Lovat L, Ourselin S, Kashin S, Wang HP, Wang WL, Haidry RJ. Artificial intelligence for the real-time classification of intrapapillary capillary loop patterns in the endoscopic diagnosis of early oesophageal squamous cell carcinoma: A proof-of-concept study. *United European Gastroenterol J* 2019; 7: 297-306. Copyright© The Authors 2019. Published by SAGE Journals.

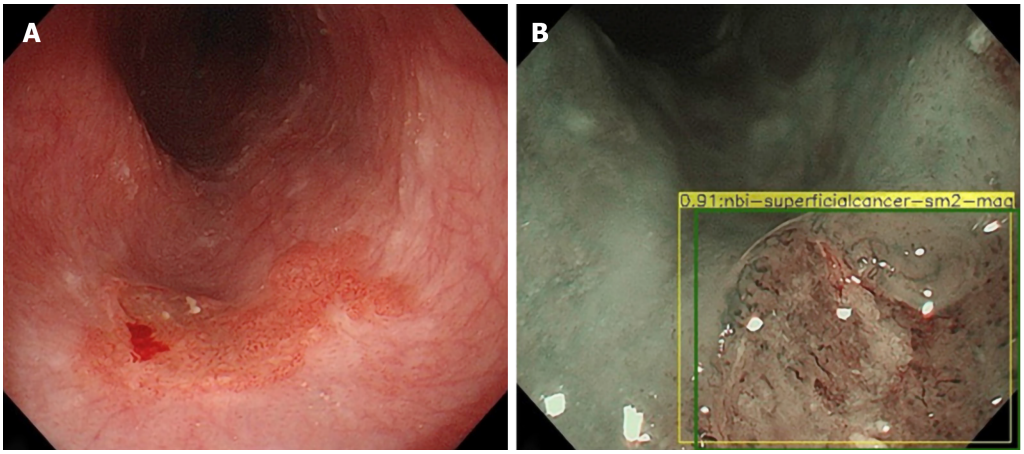

Figure 7 Esophageal squamous cell cancer diagnosed by the artificial intelligence system as superficial cancer with SM2 invasion. A and B: Citation: Nakagawa K, Ishihara R, Aoyama K, Ohmori M, Nakahira H, Matsuura N, Shichijo S, Nishida T, Yamada T, Yamaguchi S, Ogiyama H, Egawa S, Kishida O, Tada T. Classification for invasion depth of esophageal squamous cell carcinoma using a deep neural network compared with experienced endoscopists. *Gastrointest Endosc* 2019; 90: 407-414. Copyright© The Authors 2019. Published by Elsevier.

At the same time its key that AI systems avoid 'overfitting' where it performs well on training data but underperforms when exposed to new data. It needs to be able to detect early oesophageal cancer in low- and high-quality frames during real time endoscopy. This requires high volumes of both low- and high-quality training data tested on low- and high-quality testing data to reflect the real world setting during an endoscopy.

Further research is required on the use of AI in quality control in the oesophagus in order to allow endoscopists to meet the quality indicators necessary during a surveillance endoscopy as set out in many of the international guidelines. This will ensure a minimum standard of endoscopy is met.

Research in this area of AI is expanding and the future looks promising. To fulfil this potential the following is required: (1) Further development is needed to improve the performance of AI technology in the oesophagus to detect early cancer/dysplasia in BE or ESCN during real time endoscopy; (2) High quality clinical evidence from randomised control trials; and (3) Guidelines from clinical bodies or national institutes. Once implemented this will have a significant impact on this field of endoscopy.

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MINIREVIEWS

Gastrointestinal complications after kidney transplantation

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Abstract

Gastrointestinal complications are common after renal transplantation, and they have a wide clinical spectrum, varying from diarrhoea to post-transplant inflammatory bowel disease (IBD). Chronic immunosuppression may increase the risk of post-transplant infection and medication-related injury and may also be responsible for IBD in kidney transplant re-cipients despite immunosuppression. Differentiating the various forms of post-transplant colitis is challenging, since most have similar clinical and histological features. Drug-related colitis are the most frequently encountered colitis after kidney transplantation, particularly those related to the chronic use of mycophenolate mofetil, while *de novo* IBDs are quite rare. This review will explore colitis after kidney transplantation, with a particular focus on different clinical and histological features, attempting to

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clearly identify the right treatment, thereby improving the final outcome of patients.

Key Words: Inflammatory bowel disease; Kidney transplantation; Solid organ transplantation; Crohn disease; Ulcerative colitis; Mycophenolate mofetil colitis; Mycophenolate mofetil; Colitis; Cytomegalovirus

Core tip: Colitis is not common after kidney transplantation and may be related to post-transplant infection or chronic immunosuppression. Clinical and histological features may be completely different from those described in the general population. We herein discuss the epidemiology, clinical and histological features, and potential for treatment of post-transplant colitis in kidney transplantation.

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INTRODUCTION

Kidney transplantation is considered the gold standard treatment in patients affected by end-stage renal disease since it significantly improves the quality of life and patient survival compared to dialysis[1]. Although much progress has been made over the years, the management of patients during post-transplant follow-up still presents numerous difficult clinical problems. The success of a kidney transplant is related to the prevention of acute rejection, and newer immunosuppressive therapy provides a significant improvement in transplant outcomes, reducing the incidence of acute rejection[1].

However, chronic immunosuppression may increase the risk of various complications, including chronic allograft nephropathy and post-transplant infections and cancers[1-4]. In addition, kidney transplant recipients are at increased risk of gastrointestinal complications, which represent a major cause of morbidity and mortality after transplantation[5,6].

Gastrointestinal complications in kidney transplant reci-pients may be a consequence of typical infections occurring in transplant recipients, such as cytomegalovirus (CMV) infection[7-10], and of immunosuppression-mediated injury to the gastrointestinal mucosa. Post-transplant inflammatory bowel disease (IBD) may arise from an inappropriate immune response to intestinal antigens resulting in continuous intestinal inflammation[5]. Immunosuppressive therapy, which could theoretically combat this inflammatory process, may paradoxically allow for dysregulation of the intestinal immune system, finally resulting in the development of post-transplant IBD[5,11,12]. Therefore, the use of mycophenolate mofetil (MMF), which is a part of the standard immunosuppressive protocol in kidney transplantation, may increase the risk of gastrointestinal complications, including diarrhoea, gastritis and specific MMF-related colitis[6,13,14]. Furthermore, kidney transplant recipients may develop a form of *de novo* IBD despite being immunosuppressed[13,15-19].

Gastrointestinal inflammatory diseases in transplanted patients are mostly colitis and are characterized by similar symptoms but different physiopathological features[20]. A variety of clinical conditions have been described, including the following (Table 1): (1) Graft-versus-host disease (GVHD); (2) Infection-related gastrointestinal colitis (mainly CMV-derived colitis); (3) Drug-induced colitis (mainly MMF-related colitis); and (4) *De novo* IBD: Crohn's disease (CD) and ulcerative colitis (UC).

Although some studies report that the incidence of IBD in solid organ transplantation is approximately 10 times higher than that observed among the general population, particularly in liver transplant recipients[21], the occurrence of IBD in kidney transplantation is rarely reported[5].

Table 1 Clinical and histological characteristics of inflammatory bowel diseases in kidney transplantation

Disease	Clinical symptoms	Laboratory findings	Endoscopic findings	Histological findings	Treatment
Graft versus host disease	Diarrhoea, cutaneous rash	Non-specific	Oedema, erythema	High number of apoptotic cells, neuroendocrine cell proliferation	Corticosteroids reduction of immunosuppression
CMV colitis	Diarrhoea, abdominal pain, malaise, fever	Leukopenia, high level of transaminases, high PCR CMV-DNA viremia	Patchy erythema, exudates, microerosions, multiple erosions	Enterocyte apoptosis, inclusion bodies, detection of CMV on immunochemistry	Reduction of MMF, endovenous ganciclovir, oral valganciclovir foscarnet, cidofovir
MMF colitis	Diarrhoea, abdominal pain	Leukopenia	Erythema, erosions and ulcers; half of patients have normal macroscopic findings	Crypt cell apoptosis, atrophy of the crypt, crypt abscesses with eosinophil infiltrates, focal cryptitis, ulcerations and erosions	Reduction of MMF, discontinuation of MMF in severe forms
De novo IBD	Bloody diarrhoea, abdominal pain, intestinal subocclusive crisis	Elevated C-reactive protein	Patchy colitis, left-sided limited disease, pancolitis (UC), ileitis with multiple ulcers (CD)	Severe chronic inflammation with cryptitis and CD	Corticosteroids (effective), tacrolimus (low efficacy), cyclosporine (no efficacy), azathioprine (low efficacy), mesalazine/sulfasalazine (high efficacy), infliximab (limited experience)

CMV: Cytomegalovirus; PCR: Polymerase chain reaction; DNA: Deoxyribonucleic acid; MMF: Mycophenolate mofetil; IBD: Inflammatory bowel disease; UC: Ulcerative colitis; CD: Crohn's disease.

The aim of this review is to evaluate the natural history of gastrointestinal inflammatory disease in renal transplant recipients, with particular emphasis on the incidence, clinical characteristics, and potential for effective therapy. Moreover, a brief overview of the outcomes of kidney transplantation in patients with previous inflammatory bowel disease is also reported.

Literature search

The PubMed database was searched for articles by using the following terms: "chronic kidney disease", "chronic renal insufficiency", "Crohn's disease", "kidney transplantation", "ulcerative colitis", "inflammatory bowel disease", "graft-versus-host", "colitis", "mycophenolate-mofetil colitis", and "mycophenolic acid". Titles and abstracts were screened by two authors (Rossella Gioco and Massimiliano Veroux) to identify potentially relevant studies, and all potentially eligible studies were subsequently evaluated in detail by three authors (Massimiliano Veroux, Daniela Corona and Rossella Gioco) through consideration of the full text. The reference lists of retrieved articles were also searched for relevant publications. Experimental studies, clinical trials, meta-analyses, narrative reviews, and systematic reviews published in the last 20 years were included. Bibliographies of relevant articles and reviews were manually screened to identify additional studies. Studies were excluded if they were not in the English language, if they did not fit the research question, or if they had insufficient data. Initial database searches yielded 247 studies from PubMed in the last 20 years. After the evaluation of the bibliographies of the relevant articles, we evaluated 32 eligible full-text articles.

EPIDEMIOLOGY AND PATHOGENESIS OF GASTROINTESTINAL COMPLICATIONS IN KIDNEY TRANSPLANT RECIPIENTS

Very few studies have investigated the incidence of gastrointestinal inflammatory complications after kidney transplantation. A true incidence is difficult to assess due to the heterogeneity of classification and clinical manifestations. Clinical manifestations vary from diarrhoea, which is the most common symptom, to true IBD, which is largely less common. Moreover, most of the studies focused on a small proportion of patients undergoing diagnostic colonoscopy for symptomatic diarrhoea, and in most cases, the final diagnosis was a non-specific colitis, which could underestimate the true prevalence of the disease. *De novo* IBD after solid organ transplantation (SOT) is extremely rare (206 cases/100000): The majority of cases occur in liver transplant recipients, while only a few cases have been reported in renal transplant recipients[22,23].

In a review, Wörns *et al*[24] reported 44 cases of *de novo* IBD, but only 2 were detected in kidney transplant re-cipients. A more recent review[16] identified a total of 27 *de novo* IBD patients (15 patients with UC and 12 patients with CD) after renal transplantation. In a descriptive study on histological features of IBD in kidney transplant recipients, Pittman *et al*[5], among 700 kidney transplant recipients, identified 51 patients (7.2%) with gastrointestinal symptoms. Most of them (33%) were ultimately considered to have medication-related colonic injury, mainly MMF colitis, while 11 (22%) had infectious colitis, mainly from *Clostridium difficile* and CMV infections. Four (8%) patients had clinical and histopathological colitis suggesting a *de novo* IBD. In a cohort of 940 kidney transplant recipients, Dobies *et al*[25] found an IBD in 7 patients (0.7%). An additional case of *de novo* CD was recently reported by Motté *et al*[19], making for a total of 46 *de novo* histologically proven IBD cases (23 UC cases and 21 CD cases, plus 2 cases not otherwise specified) reported to date in kidney transplant recipients[5,15,19,20,22-34], including three paediatric patients[18,32]. In contrast, MMF-related colitis has a higher incidence, since it is present in up to 47% of patients undergoing colonoscopy for chronic diarrhoea[5,13,25,35].

Post-transplant *de novo* IBDs present more frequently in males, with a mean age of 35 years[5,15,18,21,22,26-35]. The main presenting symptoms are diarrhoea, abdominal pain and bright red haematochezia[5,15,18,21,22,26-34], and the mean time after transplantation to IBD presentation is 4.6 years[5,15,19,20,22-31]. Only in one patient did IBD present early, within one year after transplantation[18].

Several hypotheses have been suggested to explain the paradoxical development of *de novo* IBD in kidney transplant recipients. In the nontransplant population, IBD is generally caused by the activation of the immune system towards intestinal antigens, which may include normal intestinal microbiota[36-38]. In a kidney transplant setting, immunosuppressive therapy may provoke dysregulation of the intestinal immune environment, making it more susceptible to various insults that may damage the epithelial barrier of the intestinal mucosa, allowing prolonged exposure to luminal antigens. This exposure could lead to chronic immune stimulation and IBD, similar to what happens in non-immunosuppressed individuals who develop CD[39,40]. Moreover, a gut microbiota dysbiosis may be responsible of an increased risk of post-transplant diarrhoea and gastrointestinal complications[41,42].

Immunosuppression may increase the patient's susceptibility to opportunistic infections, such as CMV, *Escherichia coli, Campylobacter*, or *Salmonella* infections, which may trigger IBD[22,23,39,40,43,44], as demonstrated by the likely simultaneous occurrence of IBD and gastrointestinal infections[9,10].

Moreover, immunosuppressive drugs themselves may be responsible for the increased susceptibility of the gastrointestinal mucosa. Experimental studies in mice have shown that interleukin-2 (IL-2) has important inhibitory effects on T cells, and a reduction in IL-2 may provoke autoimmune colitis similar to UC[45]. The extensive use of IL-2 inhibitors such as basiliximab and tacrolimus in the induction and maintenance of post-transplant immunosuppressive therapy may therefore predispose patients to an increased risk of IBD[7,12,46], while the use of azathioprine might exhibit a protective role[11]. However, in the nontransplant population, the use of tacrolimus showed a clinical benefit in the management of IBD in the short term[47,48], suggesting that in the transplant population, the pathogenesis of *de novo* IBD is multifactorial and requires a multihit process.

Moreover, MMF and mycophenolic acid (MPA), which are two of the most effective immunosuppressants in renal transplant recipients[1], are a frequent cause of post-transplant colitis with diarrhoea[5,6,13]. MPA and MMF exposure seems to directly cause local gastrointestinal toxicity[13], which may ultimately determine apoptotic cell death and crypt damage through cytotoxic or immune-mediated mechanisms[13].

Finally, it has been suggested that steroids may have a protective role due to the likely occurrence of IBDs late in posttransplant follow-up, when the dosage of steroids is at its minimum[23,26]. Indeed, the few patients who presented IBD in the first post-transplant semester had early steroid withdrawal[22,23].

DIAGNOSIS

IBD may appear as an exacerbation of a pre-existing disease or, more rarely, as *de novo* IBD occurring in patients without any previous symptoms, and post-transplant *de novo* diseases may have a more aggressive clinical course[22,23,26]. A combination of clinical, endoscopic, and histologic features is useful to distinguish between causes of gastrointestinal symptoms affecting renal transplant recipients. The clinical manifestations are extremely varied, and patients are usually diagnosed with a form of IBD after excluding other aetiologies. In most cases, patients present symptoms such as bloody diarrhoea, abdominal cramping and bright red haematochezia.

Detailed descriptions of the clinical and endoscopic features of post-transplant IBD are limited[35]. The main feature is the presence of chronic inflammation of the mucosa, involving any tract of the digestive system in the case of CD or only the colon in the case of UC. They are both chronic intermittent diseases that can evolve into severe forms. The endoscopic pictures are patchy colitis, left-sided limited disease, pancolitis or ileal disease suggestive of CD or UC[22-31], while the presence of erythema and erosion/ulcers may be significantly associated with MMF-related colitis[35]. However, up to half of patients may have normal endoscopic findings, particularly in MMF-related colitis[35,49] or nonspecific colitis[50]. Interestingly, to date, no studies have evaluated the utility of faecal and blood markers for the detection of endoscopically active post-transplant IBD[51].

CLINICAL AND HISTOLOGICAL FEATURES

GVHD

The term GVHD refers to a clinical syndrome that occurs in transplanted patients with injury to target organs such as skin, liver, gastrointestinal tract and, more rarely, other organs[52]. It is more common in bone marrow transplantation and rarely occurs in SOT recipients[52]. Its incidence is higher in small bowel transplant recipients (5%), while in liver transplant recipients, it occurs 1 to 11 wk after transplantation, with a frequency ranging between 0.1% and 1%[53] and a mortality exceeding 75%[54]. GVHD in kidney transplant recipients is extremely rare, with only six cases reported in a recent review by Guo *et al*[55]. Clinical manifestations include diarrhoea (in the case of gastrointestinal tract involvement) and cutaneous rashes (in the case of skin involvement), with some degree of kidney function impairment[56]. The prognosis of GVHD after kidney transplantation is usually better than that GVHD following other SOTs, probably as a consequence of the fewer donor-derived lymphocytes in kidney grafts than in other solid organ grafts, and only 2 of 6 patients have died of GVHD in the kidney transplant setting[55].

The pathogenesis of GVHD is still incompletely understood, but it is probably triggered by the destruction of host tissues through several different mechanisms involving donor cytotoxic T cells, natural killer cells, cross reactivity between antigens on intestinal bacteria and the epithelium and the release of cytotoxic agents following the interaction between the host and donor cells and tissue injury[56-58]. Koyama *et al*[59] suggested that GVHD is initiated by the interaction between recipient antigen-presenting cells and donor T cells. After transplantation, these antigen-presenting cells are modified by cellular pattern signals derived from the intestinal microbiota. Donor dendritic cells in the gastrointestinal tract are activated in the colonic mucosa, resulting in the development of GVHD[59].

GVHD involving the gastrointestinal tract is characterized by crypt cell apoptosis, which is a histologic feature also described in other disorders associated with immune dysregulation and IBD, such as MMF colitis[35,60]. However, the absolute number of apoptotic cells may be significantly higher in GVHD than in MMF colitis[61]. Moreover, among the other features characterizing the histology of GVHD, there is neuroendocrine cell proliferation, which is probably a compensatory response to cell loss, periglandular inflammatory infiltrates, crypt cell cytologic atypia and histologic features of chronic inflammation similar to those described in inflammatory bowel

disease and MMF colitis[35,60-62].

Treatment of GVHD includes methylprednisolone and decreased immunosuppression with the aim of destroying activated donor-derived lymphocytes as well as deleting donor-derived lymphocytes with the host's native immune system[55].

CMV colitis

Infectious complications are the most important cause of morbidity and mortality after transplantation[1,3]. The incidence varies according to many factors, including the type of transplant, the patient's immune system and the immunosuppressive therapy. It is estimated that approximately 80% of transplant recipients develop an infection after transplantation[63], and the progressive reduction in the incidence of acute rejection has led to a significant increase in infectious diseases, particularly those associated with latent viruses such as CMV. Although the introduction of CMV prophylaxis has significantly reduced the incidence of clinically evident CMV disease in the early period after transplantation, CMV is the virus that most commonly infects patients undergoing SOT, with significant consequences on graft and patient survival. In the first year after transplantation, 50%-70% of patients experience primary infection, reactivation or reinfection[63]. When CMV infection causes significant viral replication and symptomatic illness, it may cause tissue-invasive disease with end-organ damage from the virus[64,65]. The gastrointestinal tract is most commonly affected during CMV tissue-invasive disease, resulting in oesophagitis, gastritis, enteritis, or colitis[66,67]. Diagnosis requires a biopsy obtained during oesophagogastroduodenoscopy and/or colonoscopy with histologic or culture-based evidence of CMV[66,68]. Gastrointestinal involvement during CMV infection is described in approximately 5% of patients undergoing SOT and may involve any part of the digestive tract[68], but its incidence may rise up to 25% in patients with clinical symptoms suggestive of CMV infection[68].

Indeed, CMV infects epithelial and mesenchymal cells and destroys them, causing ulcerations on the epithelial layer in different organs, including the small intestine and colon[69]. Moreover, several studies have highlighted an association between IBD and CMV, which is attributed to the virus's role in terms of both disease onset and severity[70,71]. Clinical symptoms of CMV colitis include fever, malaise and abdominal pain with diarrhoea, while laboratory findings include leukopenia, thrombocytopenia and high levels of transaminases[10,72,73].

Endoscopic lesions range from patchy erythema, exudates, and microerosions to oedematous mucosa with multiple erosions[10,72,73]. The main histologic feature of CMV colitis is increased enterocyte apoptosis, which is caused by viral infection, so it is difficult to distinguish CMV colitis from GVHD on gastrointestinal biopsy[72]. Definitive diagnosis in kidney transplant patients requires histologic findings of characteristic inclusion bodies on haematoxylin and eosin staining (Figure 1), in addition to macroscopic lesions on endoscopy[72]. Moreover, the detection of CMV in formalin-fixed tissue with immunochemistry, eventually integrated with polymerase chain reaction (PCR) of the paraffin-embedded tissue, is a highly valuable method for confirming the diagnosis of CMV colitis[74].

Serologic testing (for CMVIgG) is ineffective for diagnosing CMV disease because most cases present in a state of viral reactivation of latent virus[72]. The late CMV antigen (pp65) and quantitative CMV PCR assays have a high sensitivity for CMV viremia, but they are not good predictors for CMV tissue-invasive disease[72,73] and have a poor sensitivity (range, 48%–73%) in the detection of GI tract disease[72,73,75]. In a recent study, Durand *et al*[68] evaluated the sensitivity of quantitative PCR (qPCR) for plasma CMV deoxyribonucleic acid for the diagnosis of gastrointestinal CMV infections. Among 81 solid organ transplant recipients (liver and kidney), 20 endoscopic biopsy-proven cases of CMV of the gastrointestinal tract were identified. Overall, the sensitivity of qPCR for diagnosing CMV gastrointestinal tract disease was 85%, and the specificity was 95%. Interestingly, the sensitivity of qPCR in CMV-seronegative recipients with CMV-seropositive donors (D+/R-) was 100%, while in CMV D+/R+ recipients, it was 72.3%, probably as a consequence of the difference in immune response[68]. Indeed, in CMV R-patients, gastrointestinal CMV disease is a consequence of primary infection with high-grade viremia. In contrast, in CMV R+ patients, gastrointestinal disease follows a reactivation of CMV that could be limited by pre-existing immunity[68].

Treatment of CMV colitis includes reduction of immunosuppression and the introduction of specific endovenous antiviral drugs, such as I.V. ganciclovir (5 mg/kg BID) for a period of 10-14 d until resolution of symptoms, followed by oral valganciclovir (900 mg once a day) for 3-6 mo. High-dose valganciclovir (up to 1800 mg twice a day) and/or foscarnet and cidofovir along with immunosuppression reduction may be a treatment option for CMV colitis with ganciclovir resistance[76].

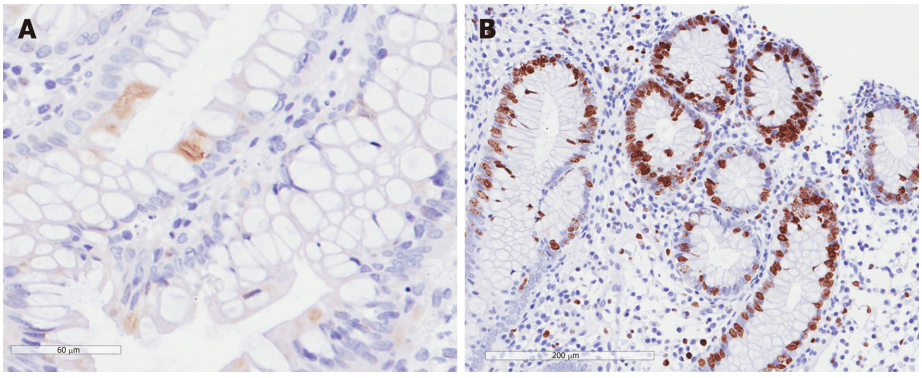

Figure 1 Cytomegalovirus colitis. A: Positivity for early cytomegalovirus antigen on immunochemistry; and B: Positivity for Ki-67 antigen on immunochemistry.

Mycophenolate mofetil-related colitis

Diarrhoea and other gastrointestinal symptoms are frequent complications after SOT, with an incidence of 12.6%, and up to 34% of cases may be related to the use of immunosuppressive drugs[19].

The introduction of MMF in the immunosuppressive regimen has led to a significant reduction in the incidence of acute rejection[1,61], although its use is associated with an increased rate of gastrointestinal complications[1,61]. It is a derivative of MPA, an antibiotic extracted from *Penicillium stoloniferum*. After oral administration, it is hydrolysed into its active metabolite, MMF. MPA inhibits the type II isoform of inosine monophosphate dehydrogenase, a key enzyme in the *de novo* synthesis of purines, causing the depletion of guanine and deoxyguanosine nucleotides, inhibiting the proliferation of T and B lymphocytes and the formation of antibodies[77].

The most common adverse effect in kidney transplant patients is watery afebrile diarrhoea, with an incidence reaching 36% in renal transplant recipients[21,78], which may persist even after drug withdrawal[21,78].

The mechanism by which MMF induces changes in the gastrointestinal mucosa is unknown, but several hypotheses have been formulated. MMF could have a direct cytotoxic effect in reducing the presence of lymphocytes in the colon and the proliferation of enterocytes, which are partially dependent on the pathway of *de novo* synthesis of purines, thus contributing to gastrointestinal toxicity, which occurs with diarrhoea. In the bowel, MMF may even cause apoptosis of lymphocytes activated following contact with luminal antigens[77-79]. In addition, MPA, having an antibacterial effect, may cause a change in the autochthonous flora of the gastrointestinal tract, which could promote the growth of anaerobic bacteria responsible for tissue damage[35]. Moreover, intestinal damage could be indirectly mediated by the immunosuppressive effect of MMF and the consequent modification of inflammatory responses[35,77-79].

Over the last few years, it has come to light that MMF can cause many gastrointestinal complications, and numerous studies have tried to determine whether MMF-related colitis shows typical histological features[5,6,13,35,51,60,61]. As MMF damage may be similar to that of IBD, it is essential to distinguish MMF colitis from *de novo* IBD since the treatment and outcome are completely different, as this could avoid unnecessary reduction of immunosuppression[13,15,35,61].

MMF colitis is defined by the presence of gastrointestinal symptoms not otherwise related to any other aetiology, with endoscopic and histological features suggesting MMF colitis, and by marked improvement or resolution of symptoms with no treatment other than the discontinuation of MMF or a 50% reduction in the initial dose of MMF[13,61]. Diarrhoea is a common symptom in MMF colitis, and up to 76.5% of patients undergoing a colonoscopy for diarrhoea have histological features of MMF colitis[11,13,35]. MMF colitis commonly presents at a later stage after transplantation, usually after 4 years[11,35], although some authors reported that MMF colitis could present two years after transplantation[13]. Renal transplant recipients present a higher incidence of MMF colitis than other organ transplant recipients[35]. The reason for that is unclear, although it may be related to the heavier immunosuppression and higher doses of MMF required after renal transplantation than after other SOTs. Moreover, MMF toxicity is more severe when it is introduced later after transplantation, and it is more frequent in patients with higher serum creatinine concentrations[80]. Moreover, tacrolimus coadministered with MMF may significantly alter enterohepatic circulation, thereby increasing contact with intestinal cells and ultimately causing colitis[6,81]. In

contrast, cyclosporin coadministered with MMF reduces the excretion of MPA metabolites, and could therefore reduce the incidence of gastrointestinal injury[82]. Finally, a significant improvement in MMF-related gastrointestinal symptoms was observed after replacement of MMF with mizoribine[83].

Common colonoscopic findings include erythema, erosions and ulcers, but half of patients have normal macroscopic findings[13,35,61].

The histological appearance of MMF colitis is characterized by the presence of architectural distortion, which resembles the appearance of chronic colitis. Therefore, the diagnosis of MMF colitis is based on specific histologic features and mainly on the exclusion of an alternative aetiology for these histological findings, such as acute colitis, IBD, and GVHD[13,35,59,60]. In their study, Selbst *et al*[61] found that MMF-induced changes were similar to those in IBDs (28%), GVHD (19%), acute colitis (16%), and ischaemia (3%). Similar findings were reported by de Andrade *et al*[6], who analysed 36 patients undergoing a colonoscopy for MMF-related diarrhoea: The most frequent histologic patterns were nonspecific colitis (31.3%), IBD-like colitis (25%), normal/near normal colitis (18.8%), GVHD-like colitis (18.8%), and ischaemia-like colitis (12.5%).

Liapis *et al*[13] evaluated colonic biopsies obtained from 43 renal transplant recipients with a clinical history of MMF administration and persistent afebrile diarrhoea. The main histological features were as follows: (1) Atrophy of the crypts assessed as absent, mild, moderate or severe; (2) Distortion of the crypts, cryptic abscesses, inflammatory infiltrates and changed numbers of eosinophils classified according to severity and extent; (3) Changed numbers of eosinophils defined as low if < 40 eosinophils per high pass filter (HPF) and high if > 40 eosinophils per HPF; and (4) Oedema, ulceration and crypts with flattened epithelium classified as absent or present.

The severity of colitis is estimated as absent, mild, moderate or severe based on the presence of eosinophils, cryptic abscesses and ulceration.

In summary, MMF colitis usually presents (1) irregular or extensive atrophy of the crypt; (2) alterations of the crypts in terms of cryptic abscesses, neutrophils, eosinophils and mucin inside the lumen of the crypt; (3) mild, moderate or severe inflammatory infiltrates, mainly plasma cells and in some cases eosinophils (> 40 per HPF); and (4) focal cryptitis, ulcerations and erosions[5,6,13,60,61]. MMF colitis presents in a more severe form in the right colon than in the left colon, probably as a consequence of longer MPA exposure in the right colon and of the diminishing compound concentration in peripheral colonic segments[13,35]. Moreover, the degree of colitis is inversely correlated with the duration of MMF administration, such that a longer therapy is associated with moderate and severe degrees of colitis[13].

As reported by other studies[5,6,59-61], the histological characteristics of MMF colitis resemble those of IBD-like conditions and GVHD. However, compared with MMF colitis and IBD-like conditions, GVHD is characterized by a higher number of apoptotic bodies[6,60] and mild to moderate inflammation with mild or absent crypt distortion[13,60]. The IBD-like pattern is mainly characterized by moderate or severe crypt abnormalities with erosion and ulcerations[13].

Increased apoptosis of crypt epithelial cells plays an important role in the pathogenesis of MMF colitis. Increased cell apoptosis in association with crypt distortion irrespective of disease activity speaks in favour of a long-acting toxic effect of MMF attributed to its pharmacodynamics[5,13,60]. Increased cell apoptosis has been correlated with MMF colitis[5,13,60,61], although the absolute numbers of apoptotic cells were significantly higher in GVHD than in MMF colitis ($P < 0.0001$)[60]. The treatment of MMF colitis includes a reduction or, in more severe forms, complete discontinuation of MMF. A 50% dosage reduction[5,61] or switch to another immunosuppressant[6] usually results in a complete resolution or in a significant improvement of symptoms in most patients, while only a minority of patients require complete discontinuation of MMF[6,60].

De novo IBD

Although IBDs are characterized by a likely autoimmune etiopathogenesis, and transplanted patients are already under immunosuppression, the incidence of IBD after SOT (up to 550 cases/100000 individuals) is approximately 10 times higher than that observed among the general population (approximately 7-10 cases/100000 individuals)[5,21-23,71]. However, *de novo* IBDs after kidney transplantation are not common, with only 46 cases reported in the literature[15,19,20,22-24,26-34]. An additional 7 cases were reported by a French multicentric study[84]. Moreover, most *de novo* IBDs occur in liver transplant recipients[16], with only 5% of SOT-related IBDs occurring in renal recipients[16]. Interestingly, a recent multicentre study of IBDs and kidney transplantation found no correlation between pre-existing autoimmune disease or

immunosuppressive treatment and IBDs before or after transplantation[84].

De novo IBDs after transplantation usually present late in the follow-up, with a mean delay time to presentation up to 91 mo[5,15,19,20,22-31,84]. Clinical manifestations of *de novo* IBDs resemble those occurring in the general population and include bloody diarrhoea and abdominal pain[5,15,19,20,22-32]. Endoscopic findings included patchy colitis, left-sided limited disease, pancolitis, and ileal disease suggestive of CD or UC[5,15,19,20,22-32]. The histological features of *de novo* CD are expansion of the lamina propria by a dense lymphoplasmacytic infiltrate with basal plasmacytosis, crypt architectural distortion, and cryptitis[5,19], while UC usually presents with chronic active colitis limited to the rectum[5].

The course of post-transplant IBD appears much more aggressive than that of IBD in the general population, and it is associated with increased mortality and difficult therapeutic management, especially due to the possible interaction between immunosuppressive drugs and IBD-specific therapy[5,15,19,20,22-32,34]. Corticosteroids may induce clinical remission of the IBD, but they are unable to maintain it as monotherapy, probably because of their failure in causing apoptosis of mature T lymphocytes, which allows chronic and acute episodes of IBD exacerbation[85]. Studies on MMF have achieved contradictory results, as in some works, MMF was unable to maintain remission in patients with IBD[86], while others showed that its administration led to an improvement of symptoms[87]. Tacrolimus and cyclosporine, although highly effective in the prevention of acute rejection after transplantation, have been proven to be ineffective in the treatment of IBD[88], although recent observations in a nontransplant population suggest that tacrolimus could have a short-term clinical benefit in the management of IBD[47,48]. Conflicting results have been obtained even regarding the use of azathioprine in maintenance therapy. Timmer *et al*[89] demonstrated that azathioprine is less effective than sulfasalazine or mesalazine due to its likely side effects, including bone marrow suppression and consequent increased susceptibility to infections in already immunocompromised renal recipients. Azathioprine should be administered as maintenance therapy only in the case of failure of a mesalazine-based therapeutic regimen or in the case of a patient who requires repeated courses of steroids[89].

Among all reported cases of *de novo* IBD, 16 occurred in kidney transplant recipients, who were successfully treated with conventional medical IBD therapy (mesalazine, cortico-steroids, and azathioprine) to achieve clinical remission. Approximately half of patients are resistant to conventional IBD therapy combined with immunosuppression[5,15,19,20,22-32].

Infliximab is a chimeric monoclonal IgG1 against tumour necrosis factor α that is used for steroid-resistant IBD. In the SOT setting, infliximab has been used in heart and liver transplantation to treat refractory IBD[31,90]. Temme *et al*[31] reported a case of steroid-refractory UC successfully treated with infliximab. Infliximab was used at a dose of 5 mg/kg body weight at week 0, 2, and 4, followed by infusions every 8 wk. After completing the infliximab regimen, the stool frequency decreased, with endoscopic resolution of the colitis. Interestingly, the authors did not observe any deterioration of graft function[31]. More recently, Garrouste *et al*[34] reported the use of anti-tumour necrosis factor α antibodies in seven kidney transplant recipients with *de novo* IBD (5 patients with Crohn's disease and 2 patients with UC). Three patients had complete remission, while in the other four patients, the disease recurred or progressed. There was no significant increase in the infection rate, and only one graft was lost. However, compared with the IBD group, in the non-IBD group, the use of infliximab resulted in a higher risk of death from infection. Although this approach has the potential to be a safe therapeutic option in patients refractory to standard therapy, the clinical experience is very limited, and other supportive data are required for this approach to be used safely in the kidney transplant setting. Approximately 20% of patients are refractory to therapy and ultimately need surgical treatment with colectomy[60,61].

OUTCOMES OF KIDNEY TRANSPLANTATION IN PATIENTS WITH INFLAMMATORY BOWEL DISEASE

The clinical course of IBD presents flares and remissions, with intestinal and extraintestinal manifestations, including kidney impairment, with AA amyloidosis and IgA nephropathy as the most common diagnoses[91,92]; these complications may even result in renal insufficiency requiring kidney transplantation[90,92,93], although renal failure is a rare complication, especially in patients with CD[94]. Age and duration of

IBD are not risk factors for developing renal failure[95]. There is an association between oxalate nephropathy and IBD since the prevalence of calcium oxalate urolithiasis is up to five-fold higher in patients with CD than in the general population[96]. Moreover, CD seems to be a likely predisposing factor for haemolytic-uremic syndrome because of recurrent gastrointestinal tract infections[97]. Very few studies have reported the outcome of kidney transplantation in patients with IBD who develop end-stage renal disease[33,90,93]. In one study, a total of 21 patients with IBD (12 patients with CD and 6 patients with UC, as well as 3 patients with disease not otherwise defined) received kidney transplantation[33,31,90,93]. An additional 28 cases were reported in a French multicentric study[85].

Schnitzler *et al*[92] reported 6 patients with IBD (5 patients with CD and 1 patient with UC) who received a kidney transplant. The female/male ratio was 5/1, and the mean age was 54.1 years. Three patients received IBD treatment before transplantation (mainly 5-ASA and steroids) and underwent ileocecal resection and fistula surgery. At the time of kidney transplantation, all patients were in clinical remission. Three patients required IBD treatment after transplantation (two CD patients were treated with steroids and 6-MP and one UC patient was treated with 5-ASA and steroids). Interestingly, among the three patients requiring treatment after transplantation, two were treated before kidney transplantation. One patient developed a post-transplant lymphoproliferative disorder, and one developed kidney graft cancer. At a median follow-up of 112.5 mo, all patients were alive, and only one patient required retransplantation[92].

In the series of Grupper *et al*[90], 12 patients with IBD (7 patients with CD and 5 patients with UC) received kidney transplantation. Kidney transplantation was more frequent in males than in females, and the median age was 48.4 years. IgA nephropathy and autosomal dominant polycystic kidney disease were the most common causes of end-stage renal disease. When compared with that in a matched control group, the rate of late rehospitalization was significantly higher in the IBD group. Moreover, patient survival was significantly lower in patients with IBD, with an estimated 5-year patient survival of 80.8% *vs* 96.8% for patients with and without IBD, respectively, with a hazard ratio for the risk of death with a functioning graft of 1.41. However, the death-censored graft survival of the IBD group was comparable with that of the non-IBD group[90]. The increased risks of rehospitalization and death were related to an increased incidence of infections, probably as a consequence of the worse nutritional status among IBD patients than non-IBD patients, as demonstrated by lower BMIs and haemoglobin levels, or as a consequence of the higher chronic immunosuppression status because of IBD-related treatments[90]. Interestingly, most patients remained in clinical remission or did not experience response deterioration after transplantation[90,92], probably as a consequence of the heavier immunosuppression in the kidney transplant population than in the liver transplant population, in which the rate of recurrence is higher[92]. However, in patients in whom IBD recurred after transplantation, the median time to flare-up after transplantation was 17 mo, and CMV infection increased the risk of recurrence[85].

CONCLUSION

Gastrointestinal diseases are common after kidney transplantation and may present with a variety of clinical and histological features. The diagnosis and management of IBD after transplantation are challenging since there are no definitive histological criteria to clearly diagnose post-transplant IBD. Indeed, many histological features may be common between different clinical forms, such as mycophenolate mofetil colitis with Graft-versus-host disease, and this could render the treatment controversial.

De novo IBD after renal transplantation should be part of the differential diagnosis in patients with chronic diarrhoea and abdominal pain, even without a previous history of gastrointestinal disease, along with infectious causes, drug-related side effects, or other comorbidities. Management of post-transplant IBD can be challenging due to the contemporary use of immunosuppressive therapy, which can increase the risk of infectious complications. Moreover, the clinical course of post-transplant IBD may be more severe than that of IBD in the general population. A better definition of clinical and histological features could help to standardize the treatment and to improve the outcome of IBD after transplantation. Due to the clinical complexity of IBD patients, a close multidisciplinary approach is necessary to achieve the best clinical outcomes of IBDs after kidney transplantation.

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MINIREVIEWS

Is vitamin D receptor a druggable target for non-alcoholic steatohepatitis?

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Abstract

Nonalcoholic steatohepatitis (NASH) is a progressed stage of non-alcoholic fatty liver disease, and available therapeutic strategies for NASH are limited. Vitamin D receptor (VDR) is proposed as a druggable target for NASH due to the discovery of vitamin D deficiency in NASH patients. To date, vitamin D supplementation has not consistently conferred expected therapeutic benefits, raising the question of whether VDR can serve as a proper drug target for NASH. It is known that VDR can interact with other ligands such as bile acids in addition to vitamin D, and its expression can be induced by fatty acids, and insulin. It has also been shown that while activation of VDR in hepatic macrophages and hepatic stellate cells resulted in attenuation of hepatic inflammation and fibrosis, activation of VDR in hepatocytes could accelerate lipid accumulation. Thus, the multiplicity of VDR ligands, together with the cell type-specificity of VDR activation, must be taken into consideration in assessing the validity of VDR being a potential druggable target for NASH treatment. To this end, we have evaluated the relationship between VDR activation and various contributing factors, such as gut microbiota, bile acid, fatty acids, and insulin, in addition to vitamin D, with an expectation that a potential drug might be identified that can elicit VDR activation in a tissue- and/or cell type-specific manner and therefore achieving therapeutic benefits in NASH.

Key Words: Vitamin D receptor; Non-alcoholic steatohepatitis; Vitamin D; Bile acids; Inflammation; Lipid metabolism

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Core Tip: Vitamin D receptor (VDR) is a nuclear receptor and proposed as a druggable target for nonalcoholic steatohepatitis (NASH) due to the discovery of vitamin D deficiency in NASH patients. However, vitamin D supplementation has not consistently conferred expected therapeutic benefits. Actually, VDR can interact with other ligands such as bile acids in addition to vitamin D, and its expression can be induced by fatty acids, and insulin. Liver is an heterogenous organ, and VDR activation in hepatic macrophages and hepatic stellate cells results in attenuation of hepatic inflammation and fibrosis, whereas activation of VDR in hepatocytes can accelerate lipid accumulation. Thus, the multiplicity of VDR ligands, together with the cell type-specificity of VDR activation, must be taken into consideration in assessing the validity of VDR being a potential druggable target for NASH treatment.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is characterized by excessive lipid accumulation in the liver. Recently, NAFLD has been redefined as "metabolic associated fatty liver disease" because of its close association with obesity, type 2 diabetes mellitus, and cardiovascular disease that are common features of metabolic syndromes[1-4]. Clinically, NAFLD presents a spectrum of symptoms ranging from non-alcoholic fatty liver (NAFL**)** and non-alcoholic steatohepatitis (NASH), to the related liver fibrosis and cirrhosis. NAFLD has become the most common liver disease worldwide, with an incidence rate of approximately 25%[5], and an average incidence rate of 7.6% in children[6]. Although in most cases NAFL is asymptomatic with no overt clinical manifestation, approximately 20%-30% of NAFL patients develop into progressive NASH. It has been estimated that NASH will become the most common indication for liver transplantation by the year 2030[7].

To date, pathogenic mechanisms underlying the development or progression of NASH remain unclear. Theories hypothesizing a "two-hits" or "multiple parallel hits" mechanism for NASH pathogenesis have been proposed, postulating the involvement of both hepatic and non-hepatic factors in the disease progression, including insulin resistance, oxidative stress, endoplasmic reticulum stress, bile acid metabolism, adipokines and gut microbiota[8-10]. In addition, genetic polymorphisms as well as epigenetic factors might also play a role in NASH development. Lifestyle modification is the first-line recommendation for NASH; thus even a modest (10%) yet sustained weight loss can confer a significant improvement in NASH and liver fibrosis[11]. In spite of lifestyle improvement, pharmacological interventions are desirable. Currently, several pre-clinical trials (phase II and III) aiming at bioenergetics, lipotoxicity, inflammation, and/or fibrogenesis are ongoing. Thus far, none of the trials have achieved desirable outcome and thus no Food and Drug Administration approved drugs are available.

Vitamin D is a lipid-soluble vitamin and its metabolite 1,25-dihydroxy-vitamin D3 [1,25(OH)2D3] functions as a steroid hormone *via* binding to vitamin D receptor (VDR). While vitamin D is mostly reported to participate in calcium/phosphate metabolism and bone homeostasis[12,13], recent studies have shown that lowered vitamin D levels are present in NAFLD/NASH patients[14-16]. Deficiency in vitamin D might result in upregulation of inflammation and oxidative stress genes in the liver (through endotoxin and toll-like receptor pathways), that are hallmarks of NASH[17,18]. Thus, attempts have been made to retard NASH progression with vitamin D supplementation.

VITAMIN D SUPPLEMENTATION IN THE TREATMENT OF NAFLD

Vitamins are small organic molecules with catalytic effects vital to health. There are six known subtypes of vitamin D with varied side chains, ranging from vitamin D2 to D7[12]. The most important vitamin subtypes in humans are D3 (cholecalciferol) and D2 (ergocalciferol). Vitamin D2 mainly comes from plants and present in fortified foods, whereas vitamin D3 is formed in the skin. Provitamin D (7-dehydrocholesterol) is converted into pre-vitamin D3 in the skin, upon exposure to ultraviolet B radiation at wavelengths between 290 and 315 nm[13]. The resultant pre-vitamin D3 is transported to the liver, where it is hydroxylated at C25 to form 25 hydroxyvitamin D3 [25(OH)D3], the major form of circulating vitamin D clinically used as a biomarker in monitoring vitamin D status. In the kidney, 25(OH)D3 is further hydroxylated at the C1 position of the A ring to form 1,25(OH)2D3, which is the most biologically active form of vitamin D.

Rodent studies have shown that prophylactic vitamin D treatment could reduce liver fat accumulation, relieve liver and intestinal inflammation, decrease the expression of fibrogenic genes, and retard the development of NASH[19,20]. In light of clinical observations that lowered level of circulating 25(OH)D3 was present in NASH patients[21-23], these animal studies provide tantalizing evidence that vitamin D supplementation might be of therapeutic value in the treatment of NASH. In a double-blinded, randomized, placebo-controlled trial, vitamin D3 treatment (50000 IU for 12 wk) resulted in improved homeostasis model assessment-insulin resistance, serum alanine transaminase (ALT), aspartate transaminase, C-reactive protein, and adiponectin, but with no effect on body weight or blood lipids[24]. In a parallel, double-blind, placebo-controlled study where NAFLD patients were randomly allocated to receiving 50000 IU vitamin D3 (n = 27) or placebo (n = 26) for 4 mo, patients receiving D3 showed significant decrease in serum malondialdehyde, indicative of attenuated oxidative stress[25]. Another prospective study showed that in 40 NASH patients with confirmed vitamin D deficiency, treatment with 20000 IU vitamin D per week for 4 wk resulted in marked improvement in hepatic steatosis[26]. These animal and clinical studies argue favorably the benefit of vitamin D supplement in ameliorating NAFL and/or NASH progression.

Conflicting results, however, have been reported about the benefit of vitamin D supplement in the management of NAFL or NASH. In a study of NASH patients with vitamin D deficiency, vitamin D3 supplementation (2100 IE for 48 wk) showed little improvement in hepatic steatosis, even though its effect in reducing plasma ALT level was significant[27]. In another prospective study, 13 patients pathologically diagnosed with NASH subjected to vitamin D3 supplementation (25 000 IU/week for 24 wk) showed no change in liver biochemistry, insulin resistance index or adipocytokine profiles before and after treatment, nor was there any change in liver histology (examined by liver biopsy) before and after a high-dose of vitamin D3 treatment[28]. More concerning was a report that patients with NAFLD developed vitamin D intolerance[29]. Thus, clinical evidence for and against vitamin D supplement are inconsistent, which may stem from the lack of clearly defined mechanisms underlying the vitamin D action.

EXPRESSION OF VDR IN THE LIVER

VDR is ubiquitously expressed with highest expression in gastrointestinal tract and endocrine tissues (https://www.proteinatlas.org/ENSG00000111424-VDR/tissue). Perhaps because hepatic expression of VDR is not robust, the physiological role of VDR in the liver was ignored for some times[30]. Nevertheless, expression of VDR is detectable in rodent livers with a context-specific expression pattern. Thus, while hepatic parenchymal cells (*i.e.*, hepatocytes) exhibited low level of the VDR mRNA, non-parenchymal cells, such as sinusoidal endothelial cells, resident macrophages [*i.e.*, Kupffer cells (KCs)], and hepatic stellate cells (HSC), expressed high levels of the VDR mRNA in rats[31]. Likewise, the VDR mRNA was readily detectable in hepatocytes, KCs, cholangiocytes, and stellates isolated from mouse livers, among them KCs showed the highest VDR expression[32]. The functional significance of VDR expression in these non-parenchymal cells in the rodent livers is largely unknown.

Varied level of VDR expression has also been noted in different stages of liver pathology (*e.g.*, NAFLD *vs* NASH). Examinations of human liver biopsy samples obtained from patients with NASH or chronic hepatitis C have detected VDR signals in parenchymal cells as well as inflammatory cells. The intensity of VDR signal in

cholangiocytes was inversely related to the severity of steatosis, lobular inflammation, and the NAFLD activity score in the NASH patients[33] An inverse relationship between the liver VDR expression and the severity of liver fibrosis and inflammation was also observed in patients with chronic hepatitis C[34]. The liver VDR expression was up-regulated in patients with NAFLD, and VDR induction is more significant in patients with simple steatosis as compared to that in NASH[33]. A decrease in liver VDR expression was also observed in a diet-induced NASH mouse model[35]. These results may suggest an upregulation of VDR expression at early stages of NAFLD, which is followed by a downregulation of VDR expression in the steatohepatitis stage.

DIFFERENTIAL ACTIVATION OF VDR IN THE LIVER

The nuclear receptor VDR is composed of six functional domains, of which the E domain (encoded by exon V and exon IX of the VDR gene) is responsible for ligand binding. The VDR not only binds to its canonical ligand vitamin D, but also binds to other ligands including secondary bile acids, especially lithocholic acid (LCA) and its metabolites (3-kitoLCA, 6-kitoLCA, *etc*.)[36]. After entering the cells, the VDR ligands are translocated into the nucleus, and the translocation induces VDR phosphorylation. The phosphorylated VDR subsequently forms a heterodimer with retinol X receptor (RXR), and the resultant VDR-RXR heterodimer promotes binding of VDR to vitamin-D-responsive element (VDRE) located in the promoter regions of the effector genes[37]. The VDR-RXR thus acts as a molecular switch that transduces the signal of VDR ligands to VDRE, and VDR ligands in turn stabilize VDR-RXR conformation[38].

"Endogenous" 1,25(OH)2D3 synthesis and VDR activation in the liver macrophages

The VDR ligand 1,25(OH)2D3 originates from two synthetic pathways, one is derived from 25(OH)D3 hydroxylation in the kidney, and the other is synthesized in activated macrophages. The 25(OH)D3 hydroxylation pathway starts in the liver where pre-vitamin D3 is hydroxylated to form 25(OH)D3 (also known as calcidiol or calcifediol), catalyzed by several cytochrome P450 isoforms (*e.g*., CYP27A, CYP2R1, CYP3A4, and CYP2J3, among which CYP2R1 is the most relevant). The resultant 25(OH)D3 is converted into 1,25(OH)2D3 in the kidney through another hydroxylation reaction catalyzed by CYP27B1. The biologically active product 1,25(OH)2D3 is released into circulation and distributed to other cells. In the case of 1,25(OH)2D3 synthesis in immune cells (*e.g*., tissue resident macrophages), expression of CYP27B1 is markedly increased in activated immune cells, leading to augmented synthesis of 1,25(OH)2D3[39-43]. This resident macrophage synthesized 1,25(OH)2D3 serves as the "local ligand" of VDR within the immune cells and exerts its function endogenously[40]. In light of the critical role of liver macrophages during inflammation and injury, it is probably not outside the realm of possibility that exposure of VDR in stressed liver macrophages with endogenous 1,25(OH)2D3 (in addition to circulating 1,25(OH)2D3) could lead to context-specific activation.

Bile acid metabolism and VDR activation in hepatocytes

Bile acids, synthesized in the liver as cholesterol (steroid) derivative, play a major role in food digestion and absorption in the intestine. The majority of bile acids are reabsorbed from the distal ileum, and the remainders enter the colon and converted into secondary bile acids by the action of gut microbiomes. A mixture of bile acids (primary, secondary, conjugated, non-conjugated) in the colon can also be reabsorbed into the liver through portal vein. Excretion and reabsorption of bile acids are the two main components of a process termed "enterohepatic circulation," which drives bile flow, eliminates certain lipids, endogenous metabolites (*e.g*., bilirubin) and toxins in the liver, facilitates absorption of lipids and lipid-soluble vitamins, and optimizes the bacteria flora in the intestine[44]. Bile acids also possess functions resembling those of hormones in modulating metabolic, endocrine and immune responses by way of signaling through their respective receptor molecules. The currently known bile acid receptor proteins include farnesoid X receptor (FXR), G protein coupled receptor, pregnane X receptor (PXR), and notably, VDR[36,45].

The ability of bile acids to activate different seemingly unrelated nuclear factors highlights the multiplicity of these hormone-like steroid derivatives. The nature of this multiplicity in bile acid binding to various nuclear factors is not fully elucidated. It is known that primary bile acids (*e.g*., CDCA and CA) can act as a ligand of the FXR and PXR. However, neither PXR nor FXR responds to 1,25(OH)2D3, the canonical ligand of VDR. On the other hand, VDR can be effectively activated by bile acids such as LCA,

glycol-LCA, and keto-LCAs, and it has been shown that LCA and 3-keto-LCA compete effectively with 1,25(OH)2D3 for binding to VDR[36]. The mechanisms underlying the bile acid- or vitamin D-driven VDR activation may be different. Thus, it is reported that while vitamin D-driven VDR activation was associated with an increase in calcium levels, bile acid-driven VDR activation was not related to calcium flux[46].

The enterohepatic circulation of the hormone-like bile acids may contribute to VDR activation in the liver and intestine. In cholestasis, LCA level is increased in the liver and intestine, and the accumulation of LCA activates VDR, which converts LCA into a less toxic intermediate for excretion[47]. It has been shown that LCA can induce CYP3A4 expression in the liver through VDR, thus participating in bile acid oxidation and mediating cell detoxification[48]. Deficiency in intestinal VDR was associated with an increase in LCA-induced liver necrosis, whereas ectopic expression of CYP3A4 in an intestine-specific VDR breakage mice ($VDR^{\Delta IEpC}$) resulted in an attenuation in LCA-induced hepatotoxicity, probably through inhibition of a bile acid transporters[49]. Studies have shown that in rats deficient in vitamin D, LCA treatment could increase serum calcium levels through VDR, in addition, LCA treatment resulted in increased expression of VDR and enhanced anti-inflammatory functions[50,51]. The increased expression of VDR upon LCA treatment was associated with activation of SIRT1/Nrf2 pathway, thus reducing NF-κB phosphorylation and IL-8 secretion[52,53]. Moreover, the ligand-activated VDR expression may be tissue-specific; thus while 1,25(OH)2D3 preferentially activates VDR in the upper intestine, LCA activates VDR in the lower intestine[46].

Bacterial metabolites also play a role in VDR function. Bacterial metabolites, such as butyrate, increases expression of intestinal VDR and inhibits the inflammatory response in mice[54]. The VDR-knockout mice showed altered intestinal microflora, with a significant decrease in lactic acid-producing bacteria, and an increase in *clostridium* and *bacteroides* in comparison of wildtype mice[55]. These data suggest an interconnection between VDR and intestinal microbiomes. Since microbiome metabolites can enter the liver through portal vein, the liver cells are exposed to various VDR ligands, which may variably affect the outcome of vitamin D supplementation in the treatment of NAFLD.

DIFFERENTIAL VDR ACTIVATION IN SUBSETS OF LIVER CELLS

The VDR homologous members, such as FXR, PXR, and constitutive androstane receptor (CAR), affect almost all aspects of the liver functions, including glycolipid metabolism and bile acid homeostasis. Because of its low expression in hepatic parenchymal cells, the functional significance of VDR in the liver has been less appreciated[56]. Nevertheless, the level of VDR expression varied widely across different regions and different cell types within the liver, thus regional activation of VDR is most likely of cell type-specific significance.

Liver macrophages are able to produce 1,25(OH)2D3 from its precursor, 25(OH)D3, and the resultant 1,25(OH)2D3 can induce macrophage differentiation through a VDR-dependent mechanism[57]. *In vitro* studies have shown that $1,25(OH)_2D_3$ treatment could lead to increased phagocytic activity of macrophages, accompanied by increased secretion of antimicrobial peptides, cathelicidin, and defensin B2/4[58,59]. VDR activation in macrophages has also been shown to induce a strong immuno-suppressive effect by inhibiting MHC class II antigens that are involved in antigen presentation[58]. Thus, activation of VDR in resident macrophages has been shown to prevent liver inflammation, steatosis, and insulin resistance in mouse liver, and protect the liver from endoplasmic reticulum stress[32,60].

HSCs activation is generally believed to be the initial stage in the progression of liver fibrosis. Activation of the VDR pathway has been shown to antagonize transforming growth factorβ/SMAD-dependent transcriptional responses of multiple pro-fibrotic genes in HSC[61]. Activation of VDR in HSC was also associated with an enhanced binding to P62 (a component of Mallory-Denk Bodies and Hyaline granules)/SQSTM 1, thus probably contributing to the retarded progression of liver fibrosis and liver cancer[62].

The VDR protein and VDR mRNA are detectable in human hepatocarcinoma HepG2 cells as well as in human primary hepatocytes. It has been reported that VDR in hepatocytes might play a role in inhibiting bile acid synthesis, thus protecting liver from injury in cholestasis[47]. Transcriptomic and metabolomic analyses of hepatocytes transfected with human VDR have revealed that 20% of the VDR responsive genes were related to metabolism of lipids (including glycerolipids and phospholipids),

uptake of fatty acids, betaine, and glycerol, suggesting a critical role of VDR in lipid metabolism in cultured hepatocytes[63-65]. In a global VDR-deficient mouse model, the livers were protected from steatosis, suggesting that VDR-mediated processes may contribute to NAFLD development[66], either due to enhanced lipid synthesis or reduced lipid turnover, or both. In hepatic-specific VDR knockout mice (generated by crossing $VDR^{flox/+}$ with albumin-Cre mice), however, the livers became more susceptible to steatosis by a high-fat diet[67]. Thus, some contributing factors, other than VDR alone, must play a role in regulating hepatic lipid metabolism under high-fat diet conditions.

One such a contributing factor is insulin resistance. Insulin resistance is a common feature associated with metabolic syndromes, including NASH. Often, insulin resistance presents hyperinsulinemia, which is linked to unbridled lipolysis leading to increased fatty acid flux into the liver and, as a consequence, augmented hepatic lipogenesis[68]. It is known that hepatic VDR expression is induced by fatty acids and insulin[64]. Thus, the influence of hyperinsulinemia on hepatic expression of VDR and its potential relationship with hepatic steatosis under high-fat diet condition requires further investigation. In addition, the possibility of any unknown side effects caused by long-term vitamin D supplementation to the liver must be carefully monitored.

CONCLUSION

Besides the critical role in regulating calcium and phosphate metabolism, VDR may also play a role in the development and progression of NASH. Since vitamin D is not the sole VDR ligand, studies with vitamin D supplementation may not adequately reflect the true action of VDR in disease progression or prevention. The controversies about the efficacy of vitamin D supplement derived from many clinical investigations may stem from two oversights, one is the contribution of non-vitamin D ligands (such as secondary bile acids) to VDR activation, and the other is cell type heterogeneity of the liver (*e.g.*, parenchymal cells *vs*non-parenchymal cells such as macrophages and HSC). The liver resident macrophages, when activated, have the capacity to synthesize biologically active vitamin D locally. This "endogenous" vitamin D, along with kidney-derived 1,25(OH)2D3 and other ligands in the circulation, can conceivably activate VDR *in situ* and modulate immune responses. Activation of VDR in HSCs is also associated with an anti-fibrogenesis process. The low level expression of VDR in parenchymal cells renders the cell insensitive to its ligands (*e.g.* bile acids and circulating 1,25(OH)2D3). However, activation of parenchymal VDR may lead to lipid accumulation in the liver (Figure 1).

Since VDR exerts a rather diverse range of effects on different cells, the current strategies that non-specifically activate VDR through vitamin D supplementation may not be proper. In addition, long-term high dose vitamin D supplementation might cause other unexpected effects. Secondary bile acids (LCA, keto-LCAs, glycol-LCA) are potentially potent ligands for hepatocyte VDR activation, and gut microbiota plays a fundamental role in the production of LCA and its metabolites. These contributing factors need to be taken into considerations in the assessment of VDR activation (or attenuation) in therapies. It is likely that activation of VDR in non-parenchymal cells can protect the liver from overwhelming immune response, whereas activation of parenchymal VDR may bring undesirable outcomes.

Physiologically, liver immune cells are quiescent with low expression of CYP27B1 and VDR, and hepatocytes are exposed to low levels of VDR ligands. The metabolic circuits in different cell types may be interconnected in forming a delicate network to maintain and support the metabolic homeostasis within the liver as well with other parts of the body. NASH patients, although often in the vitamin D deficient status, are not unusual in the situation of excessive bile acids, fatty acids, and insulin. While the non-parenchymal cells might synthesize "endogenous" 1,25(OH)2D3 through a compensatory mechanism to activate VDR *in situ*, parenchymal cells can alternatively activate VDR by non-vitamin D ligands such as LCAs. Immune cells may synthesize relatively limited 1,25(OH)2D3 to activate VDR since the process is under the control of liver 25(OH)D3 provision. However, hepatocytes are continuously exposed to VDR ligands, and may accelerate lipid accumulation in the liver under metabolically compromised conditions (*e.g.*, hyperlipidemia and hyperinsulinemia).

There is, therefore, a need to understand fully the mechanisms by which VDR is activated in a cell type-specific fashion and the associated pathophysiological consequences upon VDR activation at different stages of disease development. Tissue-specific and cell type-specific VDR agonists or antagonists are expected, which can

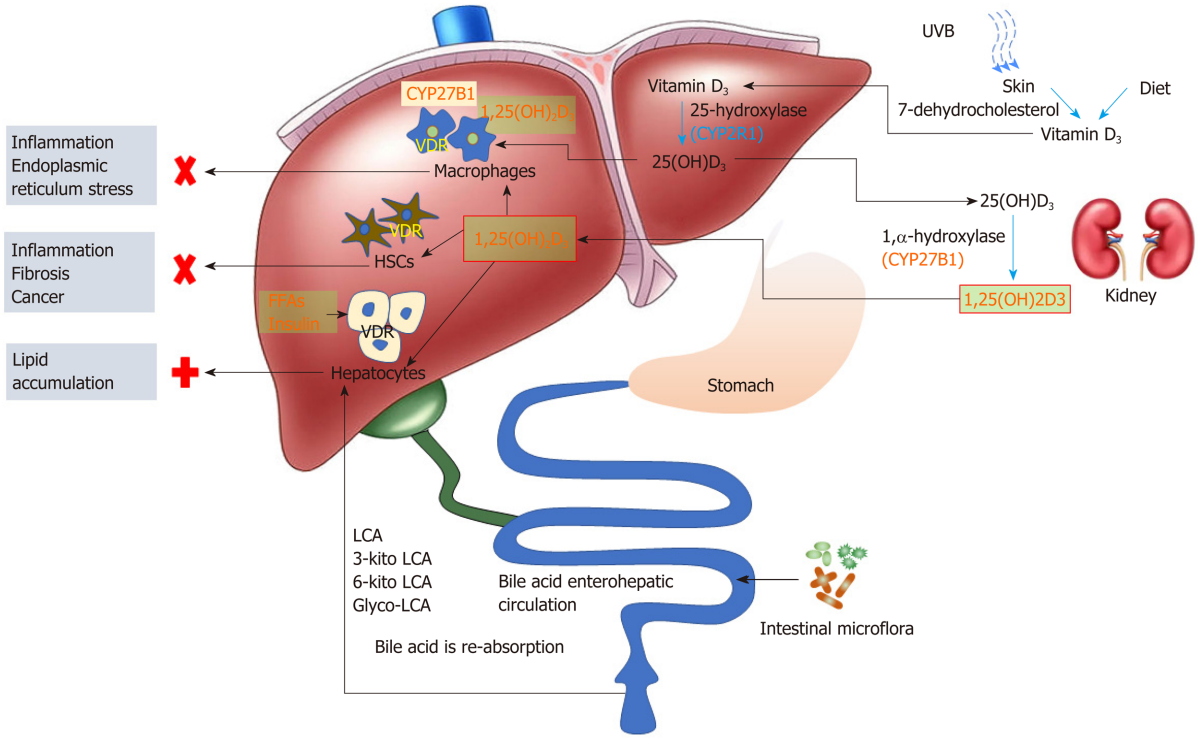

Figure 1 Expression and function of vitamin D receptor in different liver cells. Diet and skin producing pre-vitamin D (vitamin D3) is transferred to the liver, and converts into 25(OH)D3. Liver synthesized 25(OH)D3 is transported to the kidney, and converts into biochemically active 1,25(OH)2D3 *via* the catalase CYP27B1. 1,25(OH)2D3 then enters the circulation to distribute to tissues. Liver macrophages express CYP27B1, and vitamin D receptor (VDR) activation in liver macrophages can be achieved by both circulating and locally synthesized 1,25(OH)2D3; VDR in hepatocytes can be activated by circulating 1,25(OH)2D3 and gut microbiota-metabolized secondary bile acids. VDR activation in liver macrophages and hepatic stellate cells exerts anti-inflammatory and anti-fibrosis effect, respectively; whereas VDR activation in hepatocytes is supposed to contribute to lipid accumulation in the liver. LCA: Lithocholic acid; VDR: Vitamin D receptor; HSC: Hepatic stellate cells; UVB: Ultraviolet radiation B.

then selectively activate (or inactivate) VDR-mediated process in precision. Until then, more in-depth studies to define the regulatory mechanisms of VDR in NASH are merited.

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ORIGINAL ARTICLE

Basic Study

Acetyl-11-keto-β-boswellic acid inhibits proliferation and induces apoptosis of gastric cancer cells through the phosphatase and tensin homolog /Akt/ cyclooxygenase-2 signaling pathway

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Author contributions: Sun MX and He XP contributed equally to this work; Sun MX, He XP, Huang PY, Sun WH, and Hua J designed the research study; Sun MX, He XP, Huang PY, and Qi Q completed *in vivo* and *in vitro* experiments for this study; Sun WH, Hua J, and Liu GS contributed new reagents and analytic tools; Sun MX, He XP, and Hua J analyzed the data and wrote the manuscript; all authors have read and approved the final manuscript.

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Abstract

BACKGROUND

Gastric cancer is one of the most common malignant tumors of the digestive system worldwide, posing a serious danger to human health. Cyclooxygenase (COX)-2 plays an important role in the carcinogenesis and progression of gastric cancer. Acetyl-11-keto-β-boswellic acid (AKBA) is a promising drug for cancer therapy, but its effects and mechanism of action on human gastric cancer remain unclear.

AIM

To evaluate whether the phosphatase and tensin homolog (PTEN)/Akt/COX-2 signaling pathway is involved in the anti-tumor effect of AKBA in gastric cancer.

METHODS

Human poorly differentiated BGC823 and moderately differentiated SGC7901 gastric cancer cells were routinely cultured in Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Gastric cancer cell proliferation was determined by methyl thiazolyl tetrazolium colorimetric assay. Apoptosis was measured by flow cytometry. Cell migration was assessed using the wound-healing assay. Expression of Bcl-2, Bax, proliferating cell nuclear antigen, PTEN, p-Akt, and COX-2 were detected by Western blot analysis. A xenograft nude mouse model of human gastric cancer was established to evaluate the anti-cancer effect of AKBA

disclose.

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in vivo.

RESULTS

AKBA significantly inhibited the proliferation of gastric cancer cells in a dose- and time-dependent manner, inhibited migration in a time-dependent manner, and induced apoptosis in a dose-dependent manner *in vitro*; it also inhibited tumor growth *in vivo*. AKBA up-regulated the expression of PTEN and Bax, and down-regulated the expression of proliferating cell nuclear antigen, Bcl-2, p-Akt, and COX-2 in a dose-dependent manner. The PTEN inhibitor bpv (Hopic) reversed the high expression of PTEN and low expression of p-Akt and COX-2 that were induced by AKBA. The Akt inhibitor MK2206 combined with AKBA down-regulated the expression of p-Akt and COX-2, and the combined effect was better than that of AKBA alone.

CONCLUSION

AKBA inhibits the proliferation and migration and promotes the apoptosis of gastric cancer cells through the PTEN/Akt/COX-2 signaling pathway.

Key Words: Acetyl-11-keto-β-boswellic acid; Gastric cancer; Cell proliferation; Apoptosis; Cyclooxygenase-2; Tumor xenograft

Core tip: Cyclooxygenase-2 is involved in the carcinogenesis and development of gastric cancer, and is closely related to its prognosis. Acetyl-11-keto-β-boswellic acid is a promising drug for gastric cancer therapy. It inhibits the proliferation and induces the apoptosis of gastric cancer cells, possibly *via* mechanisms associated with down-regulation of cyclooxygenase-2 expression through the phosphatase and tensin homolog/Akt signaling pathway.

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INTRODUCTION

Gastric cancer is the fourth most common cancer and second leading cause of cancer-related mortality in the world[1]. It is a multifactorial disease characterized by being highly drug resistant, and apoptotic deficiency could be the obstacle that hampers the efficacy of most anti-cancer agents. Currently, the main treatments include surgery, radiotherapy, and chemotherapy. However, the prognosis of gastric cancer is still poor; thus, it is necessary to find new drugs to treat gastric cancer.

Acetyl-11-keto-β-boswellic acid (AKBA) is a pentacyclic terpenoid extracted from the gum ayurvedic therapeutic plant *Boswellia serrata*. The molecular structure of AKBA is shown in Figure 1. AKBA is anti-inflammatory agent that exhibits potent cytotoxic activities against various types of tumors, such as hepatocellular carcinoma[2], glioblastoma[3], prostate cancer[4], lung carcinogenesis[5], colon cancer[6,7], and leukemia[8,9]. However, the efficacy and mechanism of action of AKBA in treating gastric cancer have not been studied. This study is the first attempt to explore the efficacy and mechanism of action of AKBA in gastric cancer.

Cyclooxygenase (COX) catalyzes the formation of prostaglandin and other eicosanoids from arachidonic acid, which has been considered as a potential target for the prevention and treatment of cancer. It tends to be highly expressed in inflammation and tumor tissues but is undetectable in the majority of normal tissues[10-12]. COX-2, the mitogen-inducible isoform, is constitutively expressed in gastric cancer. It has been shown that overexpression of COX-2 is directly or indirectly involved in the carcinogenesis and development of gastric cancer, and is closely

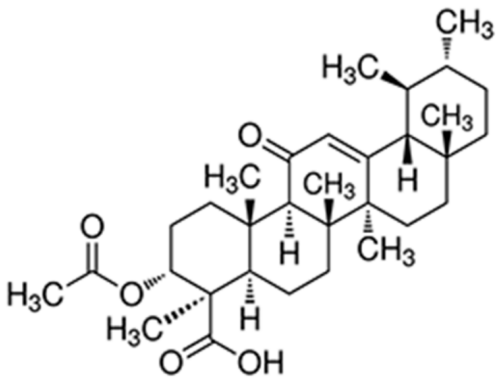


Figure 1 Molecular structure of acetyl-11-keto-β-boswellic acid.

related to its prognosis[13]. However, the molecular mechanisms underlying the aberrant expression of COX-2 in gastric cancer remain unclear. Regulation of COX-2 expression depends on different cellular pathways, involving both transcriptional and post-transcriptional mechanisms[14]. Among all upstream regulatory factors, activation of the PI3K/Akt pathway is important for COX-2 up-regulation[15-18]. PTEN (phosphate and tension homology deleted on chromosome ten) is a tumor suppressor with phosphatase activity that can regulate cell survival, proliferation, and energy metabolism[19,20]. Many tumor tissues present with deficiency of PTEN[21]. PTEN acts as a lipid phosphatase to dephosphorylate phosphatidylinositol (3-5)-trisphosphate, antagonizing the PI3K/Akt pathway[22]. Recent studies have demonstrated that the PTEN/Akt pathway can regulate COX-2 expression in human cervical cancer, gallbladder cancer, and colorectal cancer[23-25].

In this study, human gastric cancer cell lines BGC823 and SGC7901, in which COX-2 was found to be overexpressed[10,13,26,27], were used to investigate the anti-tumor effects of AKBA on human gastric cancer *in vitro* and *in vivo*. Additionally, the expression of proliferating cell nuclear antigen (PCNA), Bcl-2, Bax, COX-2, PTEN, and p-Akt was detected to elucidate the possible mechanism underlying the anti-tumor effects of AKBA against gastric cancer.

MATERIALS AND METHODS

Reagents

AKBA ($C_{32}H_{48}O_5$, CAS: 67416-61-9, purity: > 98%), dimethylsulfoxide (DMSO), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylformazan (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, United States). Roswell Park Memorial Institute (RPMI)-1640 medium, fetal bovine serum, and penicillin/streptomycin were purchased from Gibco BRL (Grand Island, NY, United States). Hoechst 33342 and apoptosis detection kit were purchased from Beyotime Institute of Biotechnology (Nantong, China). PTEN inhibitor bpv (HOpic) and Akt inhibitor MK2206 were purchased from Selleck Chemicals (Houston, TX, United States). Other reagents were of analytic grade and obtained from Nanjing Chemical Reagent Co. (Nanjing, China), unless otherwise described. AKBA was dissolved in DMSO as a 2 mmol/L stock solution and stored at -20 °C.

Cell culture and treatments

Human poorly differentiated BGC823 and moderately differentiated SGC7901 gastric cancer cell lines were obtained from Shanghai Institute of Cell Biology (Shanghai, China). All cells were routinely cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. All the cells were kept at 37 °C in a humidified atmosphere of 5% CO_2.

Methyl thiazolyl tetrazolium (MTT) assay

BGC823 and SGC7901 cells (4000-5000/well) were seeded into 96-well plates and incubated overnight in a 5% CO_2 atmosphere at 37 °C. The following day, the gastric cancer cells were treated with different concentrations of AKBA (0, 10, 20, 30, 40, and 50 μM) in serum-free conditions for 24, 48, or 72 h at 37 °C. Subsequently, 200 μL of

MTT solution (0.5 mg/ML) was added to each well and incubated for 4 h at 37 °C. DMSO (200 μL) was then added to each well. The proliferation-inhibitory effects of each combination were assessed using a microplate reader (MJ Research Inc.) at 570 nm.

Flow cytometry

To detect the apoptosis rate of gastric cancer cells, we used the Annexin V fluorescein isothiocyanate (FITC) Apoptosis Detection Kit to measure apoptosis *via* flow cytometry. BGC823 and SGC7901 cells were plated in six-well plates at 2×10^5 cells per well. All the cells were treated with different concentrations of AKBA (0, 20, 30, or 40 μM) for 48 h. Detached cells were collected, combined with the trypsinized attached cells, and centrifuged at 1400 r/min, 4 °C, for 10 min. Cells were re-suspended in 1 mL of binding buffer and allowed to react with 10 μL of FITC-labeled Annexin V and 10 μL of PI for 15 min at room temperature in the dark. Afterward, the cells were analyzed on a flow cytometer (Becton Dickinson, San Jose, CA, United States). Apoptosis was assessed by Annexin V-FITC and PI staining.

Western blot analysis

BGC823 and SGC7901 cells were grown to sub-confluence in 100-mm dishes and cultured in serum-free medium for 24 h. The cells were treated with different concentrations of AKBA (0, 20, 30, or 40 μM) in serum-free conditions for 48 h. In another experiment, the cells were pre-treated with the PTEN inhibitor bpv (HOpic, 17.4 ng/ML) or Akt inhibitor MK2206 (38.4 ng/ML) for 30 min and then with AKBA (40 μM) for 48 h. The extraction of proteins from cells and Western blotting were performed as described previously[28,29]. Antibodies used include rabbit anti-PCNA, anti-Bcl-2, anti-Bax, anti-PTEN, anti-COX-2, and anti-reduced glyceraldehyde-phosphate dehydrogenase (GAPDH) (Abcam Inc., Cambridge, MA, United States), as well as anti-phospho (p)-Akt and anti-Akt (Sigma–Aldrich). Horseradish peroxidase-conjugated goat anti-rabbit IgG purchased from Abcam was used as secondary antibody. The relative expression of PCNA, Bcl-2, Bax, PTEN, and COX-2 was normalized to that of GAPDH. P-Akt was normalized to the total Akt level.

Wound healing assay

A wound healing assay was performed as described previously[30]. BGC823 and SGC7901 cells were seeded in six-well plates (7×10^5–8×10^5 cells/well) to produce a confluent monolayer. The cell monolayer was scratched with a pipette tip and washed with phosphate-buffered saline. Cells were then incubated with different concentrations (20 μM) of AKBA. Scratch wounds were photographed under a phase contrast microscope (Leica, Nussloch, Germany) at 0, 48, and 72 h post treatment, respectively.

Anti-cancer activity in mice

The effect of AKBA was further evaluated in nude mice bearing xenografts of BGC823 and SGC-7901 cells. The use of all experimental animals was in compliance with the protocols set by Nanjing Medical University Institutional Animal Care and Use Committee. Female athymic BALB/c (nu+/nu+) mice (n = 20), aged 5 wk, were purchased from the Department of Laboratory Animal Center of Nanjing Medical University (Nanjing, China). The mice were kept in a specific pathogen-free conditional microenvironment (temperature 24 °C, humidity 60 ± 10%, and 12 h/12 h light/dark cycle). Then, 0.1 mL of cell suspension containing 5×10^6 – 6×10^6 BGC823 or SGC7901 cells was subcutaneously injected into the axillary space of the mice. When tumor volume reached approximately 100 mm^3, mice were randomly divided into different groups (n = 5): AKBA (100 mg/kg) or control (equal volume of normal saline). Mice were observed daily for clinical symptoms. Tumor volume and body weight were measured every 2 d. The tumor volume was calculated as (shortest diameter)^2 × (longest diameter) × 0.5. At the end of treatment, nude mice were intraperitoneally injected with 3% pentobarbital sodium and were euthanized by excessive anesthesia at a dose of 90 mL/kg. The tumor tissue was weighed and excised for Western blot analysis.

Statistical analysis

All data from at least three independent experiments are represented as the mean ± SD and statistically analyzed by two-tailed Student's t-test or one-way analysis of variance with Dunnett's multiple comparison tests. Statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, United States). $P < 0.05$

was considered statistically significant.

RESULTS

AKBA inhibits proliferation and induces apoptosis of gastric cancer cells

MTT assay was performed to evaluate the survival of gastric cancer cells exposed to different therapeutics (0–50 μM for 24, 48, and 72 h). AKBA significantly inhibited the proliferation of BGC823 and SGC7901 cells in a dose- and time-dependent manner (Figure 2A and B). After 72 h of treatment with 50 μM AKBA, the proliferation of BGC823 and SGC7901 cells was almost completely blocked.

Annexin V-FITC/PI assay was also conducted to investigate the apoptosis induced by AKBA on the basis of flow cytometry. In the dual-parameter fluorescent dot plots, early apoptotic cells stained with Annexin V (Annexin V+/PI−, Q3), and late apoptotic cells stained with Annexin V and PI (Annexin V+/PI+, Q2) were counted (Figure 2C). AKBA induced the apoptosis of gastric cancer cells in a dose-dependent manner (Figure 2D).

To study the effect of AKBA on cell proliferation and apoptosis, the expression of PCNA (involved in proliferation), Bcl-2, and Bax (involved in apoptosis) in BGC-823 and SGC-7901 cells was evaluated by Western blot analysis. After 48 h of AKBA treatment, the expression of Bax was up-regulated and that of Bcl-2 and PCNA was downregulated in a dose-dependent manner in BGC-823 and SGC-7901 cells (Figure 2E-G). All of these data indicated that AKBA inhibits the proliferation and induces the apoptosis of gastric cancer cells.

Effects of AKBA on expression of PTEN, COX-2, and p-AKT in BGC-823 and SGC-7901 cells

PTEN, COX-2, and p-Akt expression in gastric cancer cells was detected by Western blot analysis. AKBA dose-dependently inhibited COX-2 expression and p-Akt expression, and increased PTEN expression in BGC-823 and SGC-7901 cells (Figure 3).

Effects of PTEN/Akt inhibitor on AKBA-induced downregulation of COX-2 expression in gastric cancer cells

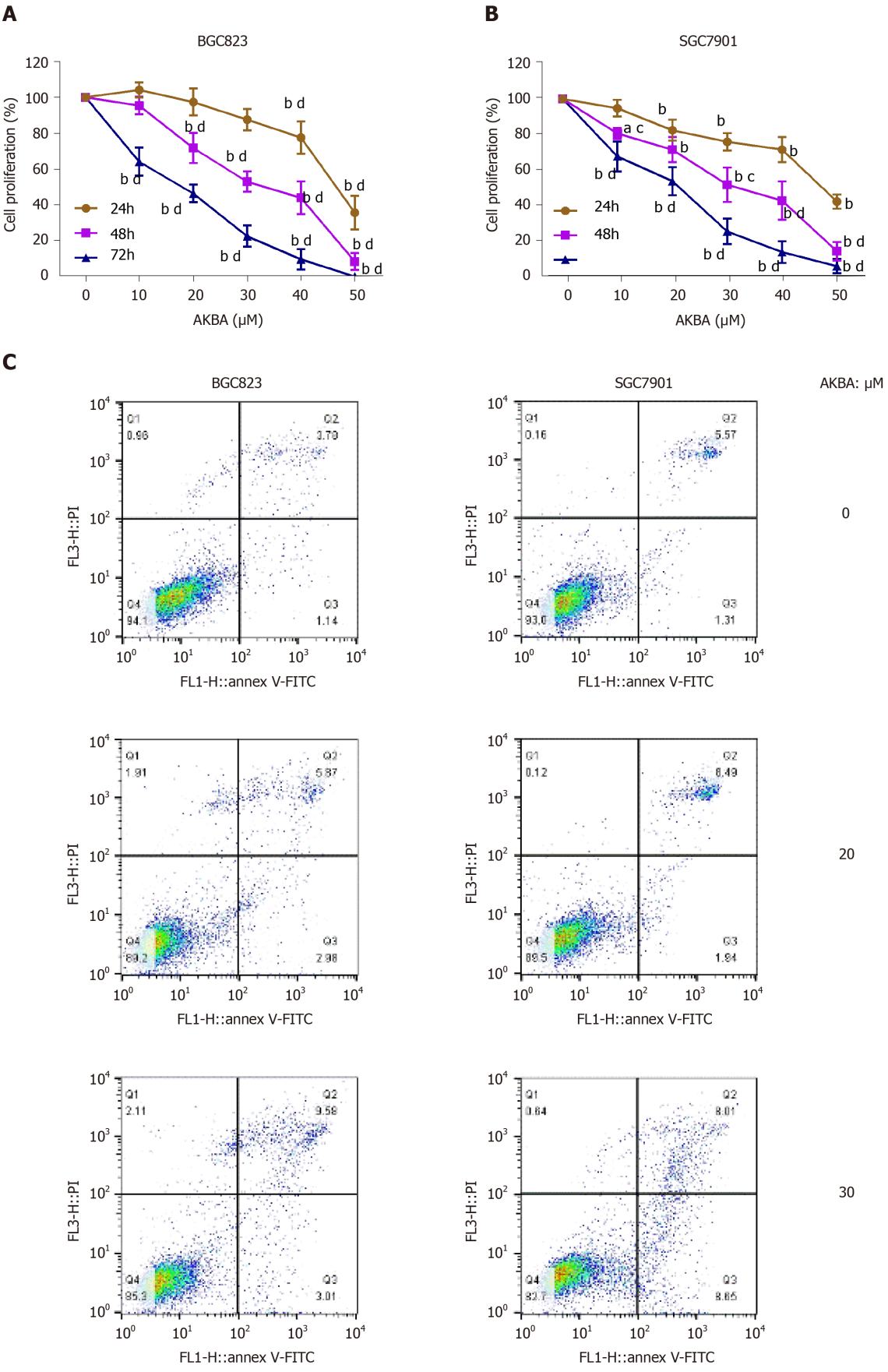
To elucidate the signaling pathway involved in the down-regulation of COX-2 expression induced by AKBA, further studies were designed to determine the effect of PTEN and Akt inhibitors on COX-2 expression in gastric cancer cells. The PTEN inhibitor bpV (HOpic) blocked the high expression of PTEN induced by AKBA and reversed the low expression of p-Akt and COX-2 inhibited by AKBA (Figure 4). The Akt inhibitor MK2206 combined with AKBA downregulated the expression of p-Akt and COX-2, and the combined effect was better than that of AKBA alone. These results collectively indicated that AKBA elicited inhibition of proliferation and induction of apoptosis of gastric cancer cells may be mediated by down-regulation of COX-2 expression through the PTEN/Akt signaling pathway.

AKBA inhibits cell migration and wound closure of gastric cancer cells

We further examined the effect of AKBA on cell migration after monolayer wounding. When BGC823 and SGC7901 cell monolayers were wounded and treated with AKBA (20 μM) for 48 or 72 h, the AKBA-treated groups closed the gaps at a slower pace at 48 and 72 h , compared with the control group (Figure 5).

AKBA inhibits gastric xenograft tumor growth in vivo

The effect of AKBA on BGC823 and SGC7901 cell xenografts was examined (Figure 6). Twenty nude mice implanted with BGC823 or SGC7901 cells were treated daily with AKBA for 2 wk. No deaths occurred within that period. The average tumor volume of nude mice treated with AKBA was markedly lower than that in the control nude mice (Figure 6A and B). There was no significant difference in body weight between the control and AKBA-treated nude mice (Figure 6C). COX-2 protein was overexpressed in gastric cancer tissues of control nude mice (Figure 6D and E). The results indicated that AKBA reduced the expression of COX-2 in the tumor tissues of nude mice, suggesting that AKBA inhibits tumor growth in nude mice by down-regulating the expression of COX-2.


A
BGC823
Cell proliferation (%)
AKBA (μM)
24h
48h
72h
B
SGC7901
C
BGC823
SGC7901
AKBA: μM
0
20
30
FL3-H::PI
FL1-H::annex V-FITC
Q1 0.96
Q2 3.79
Q4 94.1
Q3 1.14
Q1 0.16
Q2 5.57
Q4 93.0
Q3 1.31
Q1 1.91
Q2 9.87
Q4 88.2
Q3 2.98
Q1 0.12
Q2 8.49
Q4 88.5
Q3 1.84
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Q4 82.7
Q3 8.65

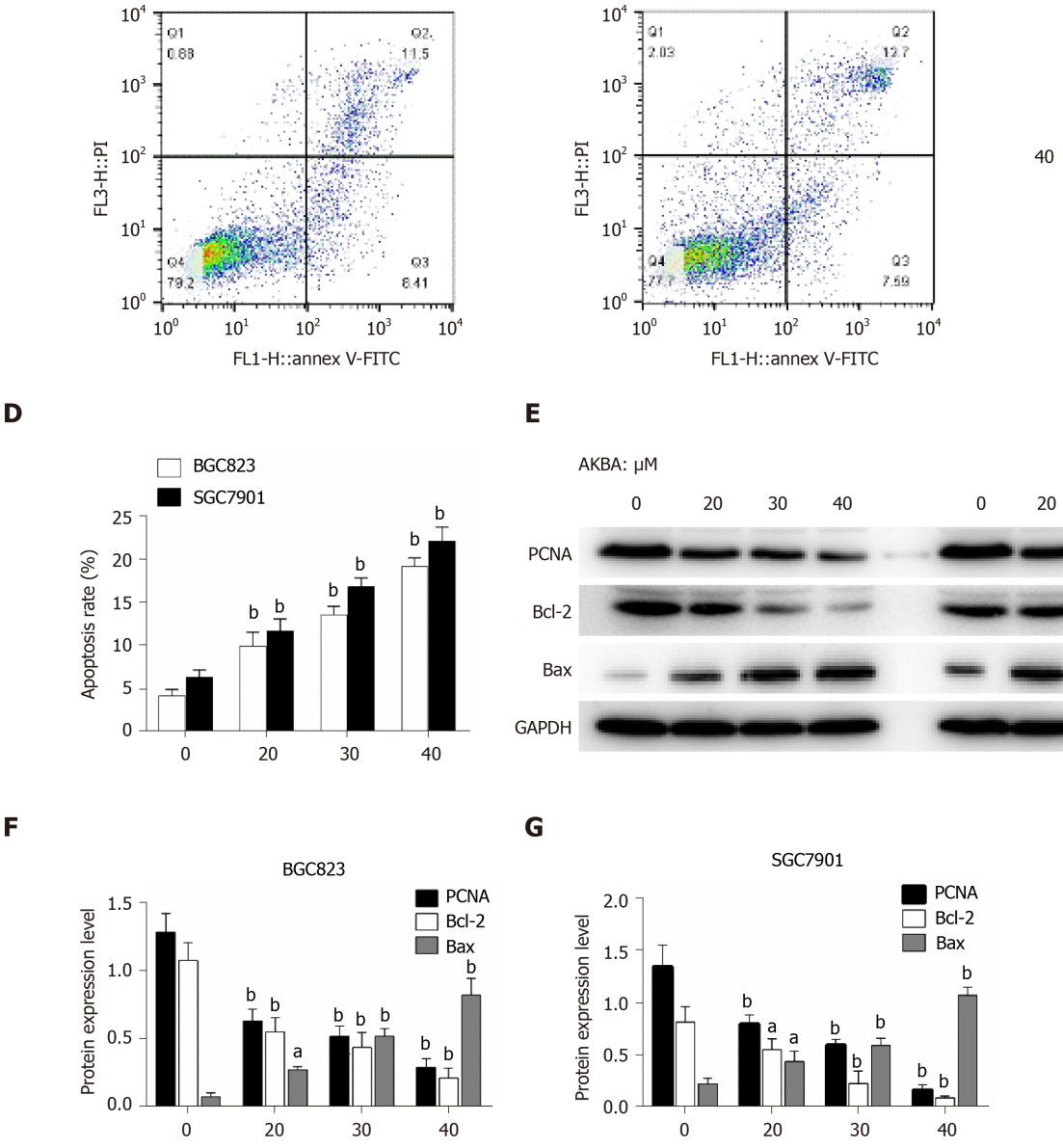

Figure 2 Acetyl-11-keto-β-boswellic acid inhibits the proliferation and induces the apoptosis of gastric cancer cells. A and B: Dose- and time-dependent effects of acetyl-11-keto-β-boswellic acid (AKBA) on BGC823 and SGC7901 cell proliferation; C: Flow cytometry-based annexin V-FITC/PI labeling of apoptotic cells; D: Histogram showing apoptosis rates; E: Expression levels of proliferating cell nuclear antigen (PCNA), Bcl-2, and Bax in BGC823 and SGC7901 cells measured *via* Western blot analysis after AKBA treatment; F and G: Histograms showing the relative expression levels of PCNA, Bcl-2, and Bax compared with GAPDH in BGC823 and SGC7901 cells. Each data point represents the mean ± SD from three independent experiments. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control (0 μM). $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* control (24 h). AKBA: Acetyl-11-keto-β-boswellic acid; PCNA: Proliferating cell nuclear antigen; GAPDH: Glyceraldehyde-phosphate dehydrogenase.

DISCUSSION

Gastric cancer is one of the most common malignancies with a high mortality rate. COX-2 is expressed in gastric cancer and is a factor involved in cancer cell proliferation and apoptosis, cancer invasiveness, and metastasis[31]. Studies have shown that COX-2 inhibition by selective COX-2 inhibitors or siRNA suppresses cell growth and leads to apoptosis in human gastric adenocarcinoma cells[29,32]. In this study, we demonstrated for the first time that AKBA can downregulate the expression of COX-2 *via* the PTEN/Akt signaling pathway in gastric cancer cells, and consequently induces the apoptosis and inhibits the proliferation of gastric cancer cells.

Previous studies have confirmed that AKBA can inhibit the proliferation and enhance the apoptosis of pancreatic cancer cells *in vitro* by reducing COX-2 expression level[33]. The results of those studies are consistent with our finding that AKBA can significantly inhibit COX-2 expression in gastric cancer cells. However, the molecular mechanism by which AKBA down-regulates the expression of COX-2 in gastric cancer cells remains unknown.

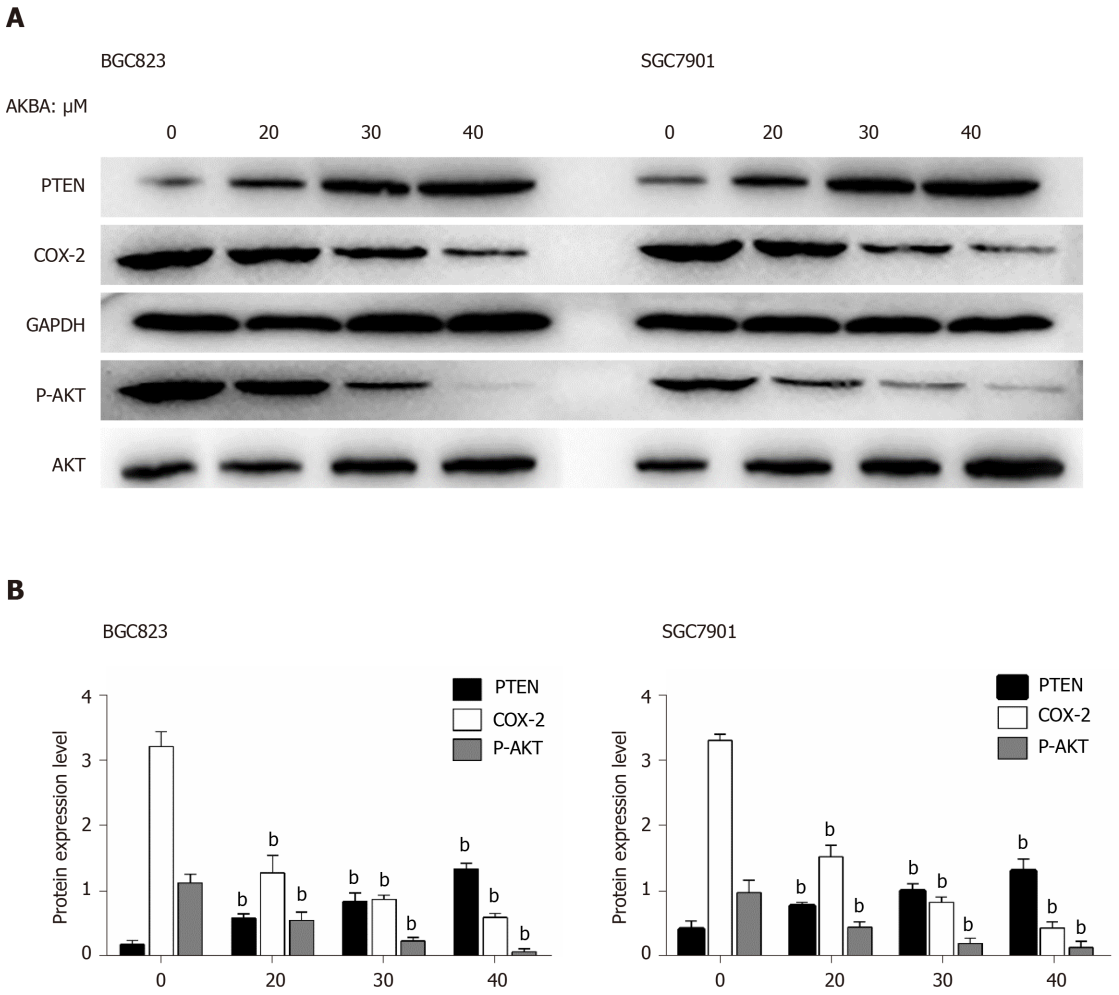

Figure 3 Effects of acetyl-11-keto-β-boswellic acid on the expression of phosphatase and tensin homolog, cyclooxygenase-2, and p-Akt in gastric cancer cells. A: Expression levels of phosphatase and tensin homolog (PTEN), cyclooxygenase (COX)-2, and p-Akt in gastric cancer cells measured *via* Western blot analysis; B: Histograms showing the relative expression levels of PTEN and COX-2 compared with GAPDH, and p-Akt compared with Akt in gastric cancer cells. Each data point represents the mean ± SD from three independent experiments. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control (0 μM). AKBA: Acetyl-11-keto-β-boswellic acid; PTEN: Phosphatase and tensin homolog; COX-2: Cyclooxygenase-2; GAPDH: Glyceraldehyde-phosphate dehydrogenase.

Numerous studies have confirmed that the PI3K/Akt signaling pathway can induce apoptosis in a variety of cancer cells[34-37]. COX-2 expression can be induced by activating the PI3K/Akt signaling pathway in gastric cancer cells, thereby regulating the proliferation of gastric cancer cells[28]. PTEN deletion increases the activation of the PI3K/Akt pathway[22,38]. When the PTEN gene is mutated or lost, the Akt signaling pathway is activated, resulting in the proliferation of cells to form tumors[39]. miR-21 promotes cell proliferation by activating PTEN/Akt signaling in cancer cells[40]. CD2 selectively induces the expression of COX-2 through PTEN-mediated PI3K/Akt activation[41]. The selective COX-2 inhibitor celecoxib inhibits tumor growth *via* the PTEN/PI3K/Akt pathway in an H_{22} murine hepatocarcinoma model[17]. Our results are consistent with those previous findings. Our results showed that AKBA can effectively inhibit the proliferation and migration of gastric cancer cells and induce their apoptosis. Additionally, AKBA significantly inhibited the expression of PCNA, Bcl-2, and p-Akt, and increased Bax and PTEN expression in gastric cancer cells. To clarify the molecular mechanisms of the anti-cancer effect of AKBA on gastric cancer, we examined ethe xpression of proteins related to the PTEN/Akt/COX-2 pathway *in vitro* (Figure 7). The PTEN inhibitor bpv (Hopic) blocked AKBA-induced PTEN expression, and reversed AKBA-induced downregulation of p-Akt and COX-2 expression. The Akt inhibitor MK2206 combined with AKBA downregulated the expression of p-Akt and COX-2, and the combined effect was better than that of AKBA alone.

In vitro data show that AKBA inhibits the occurrence and development of gastric cancer by inducing apoptosis of gastric cancer cells. Our animal experiments verified the findings of *in vitro* experiments. Previous research confirmed that AKBA has an anti-tumor effect in nude mice[2-4,33,42,43]. Our results indicate that the tumor size of

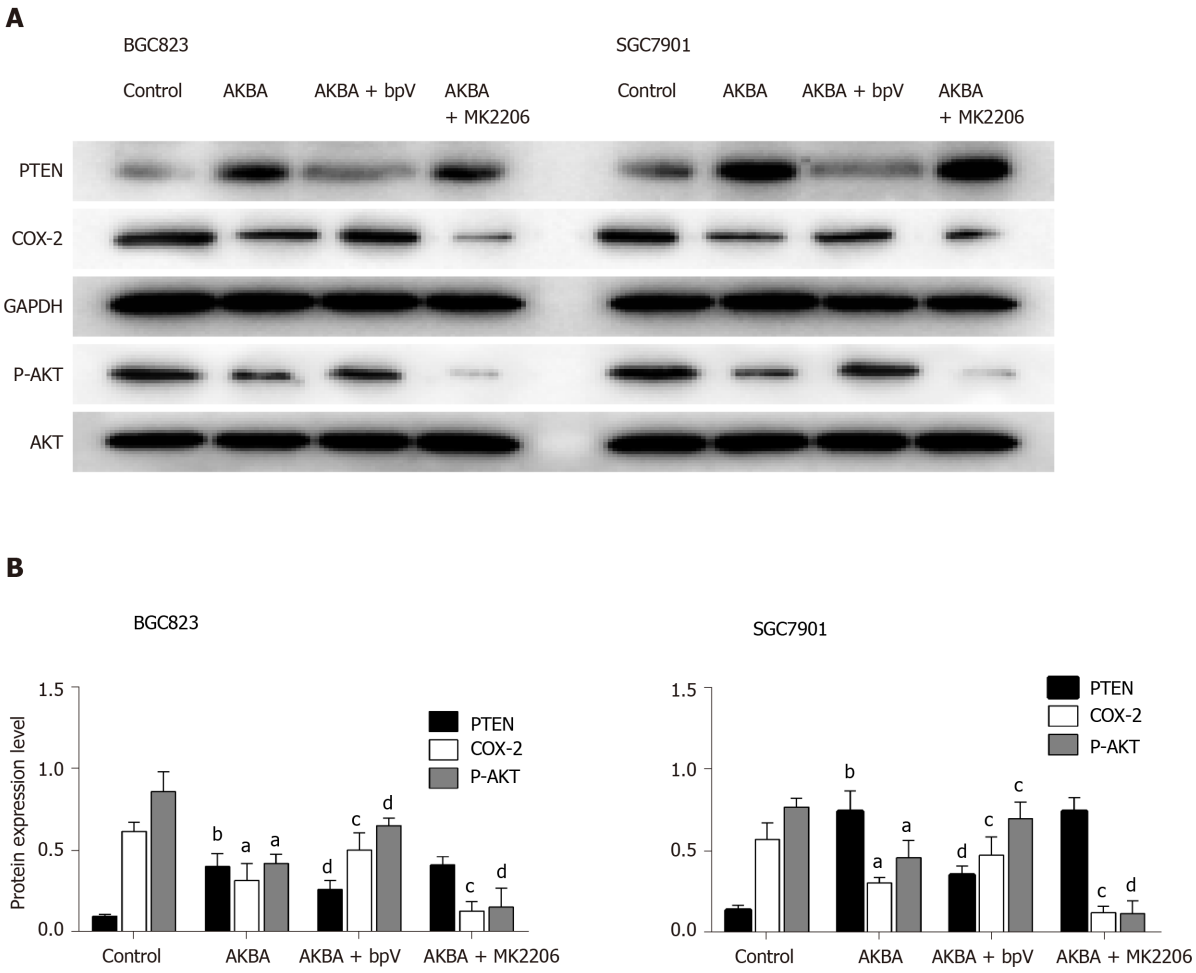

Figure 4 Effects of phosphatase and tensin homolog /Akt inhibitor on acetyl-11-keto-β-boswellic acid-induced down-regulation of cyclooxygenase-2 expression in gastric cancer cells. Gastric cells were pre-treated with the phosphatase and tensin homolog (PTEN) inhibitor bpv (HOpic, 17.4 ng/mL, A and B) or Akt inhibitor MK2206 (38.4 ng/mL, C and D) for 30 min and then treated with acetyl-11-keto-β-boswellic acid (40 μM) for 48 h. A: Expression of PTEN, cyclooxygenase (COX)-2, and total and phosphorylated forms of Akt in gastric cancer cells detected by Western blot analysis. B: Histograms showing the relative expression of PTEN and COX-2 compared with GAPDH, and phospho-Akt (p-Akt) compared with total Akt, respectively. Each data point represents the mean ± SD from three independent experiments. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* Control; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* AKBA. AKBA: Acetyl-11-keto-β-boswellic acid; PTEN: Phosphatase and tensin homolog; COX-2: Cyclooxygenase-2; GAPDH: Glyceraldehyde-phosphate dehydrogenase.

AKBA-treated nude mice is significantly smaller compared to the control group. AKBA significantly inhibits tumor growth in xenograft nude mice without causing significant weight loss, mortality, or other adverse effects.

CONCLUSION

In summary, these results suggest that AKBA inhibition of proliferation and induction of apoptosis of gastric cancer cells may be mediated by down-regulation of COX-2 expression through the PTEN/Akt signaling pathway.

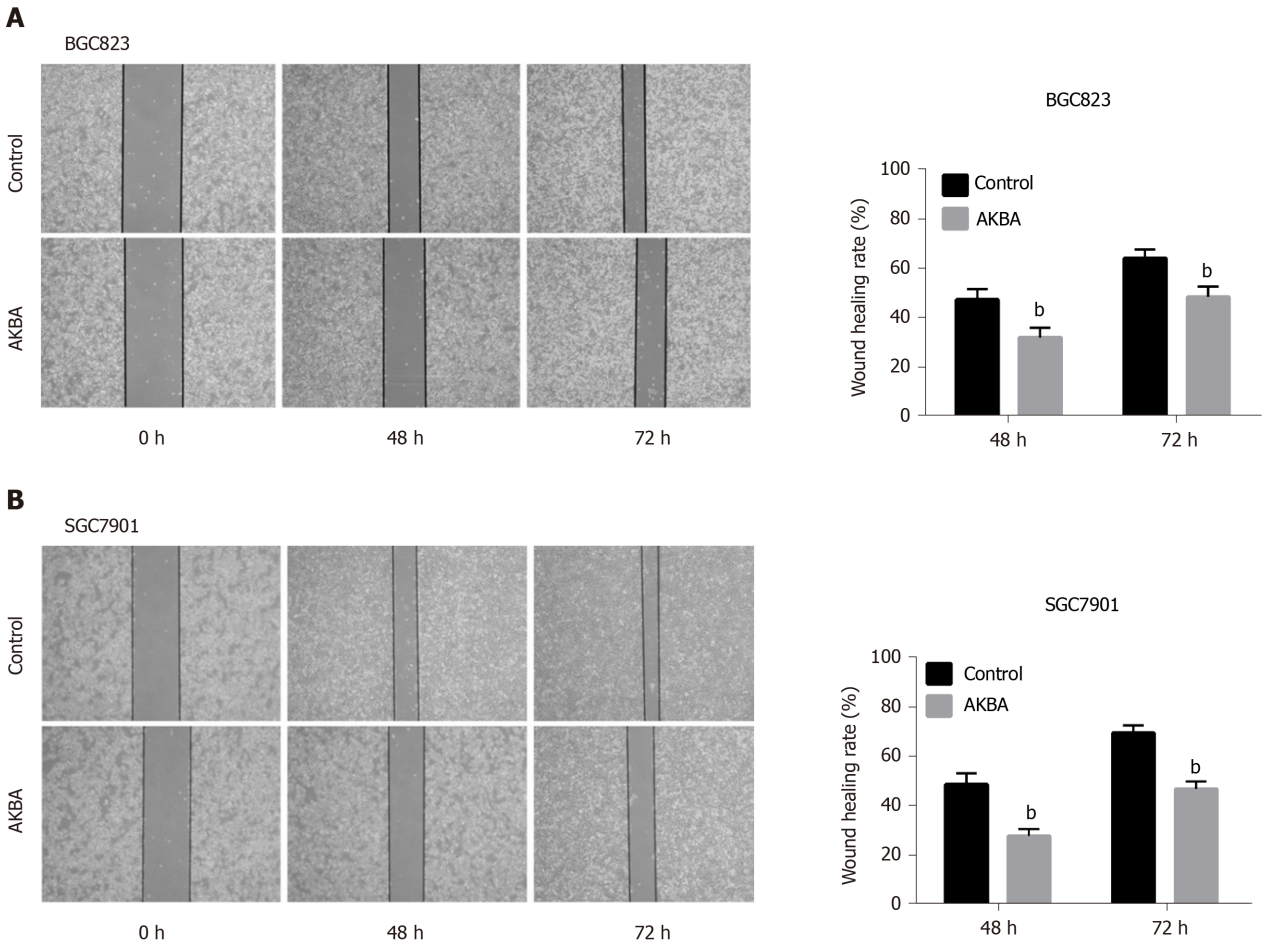

Figure 5 Acetyl-11-keto-β-boswellic acid inhibits the monolayer wound healing of gastric cancer cells. A and B: Phase micrographs of BGC823 and SGC7901 cells at various times after monolayer wounding (original magnification, × 100); C and D: Quantification of cell migration using monolayer wound-healing assay. The gastric cancer cells were treated without (control) or with 20 μM acetyl-11-keto-β-boswellic acid. Each data point represents the mean ± SD from three independent experiments. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control (0 μM). AKBA: Acetyl-11-keto-β-boswellic acid.

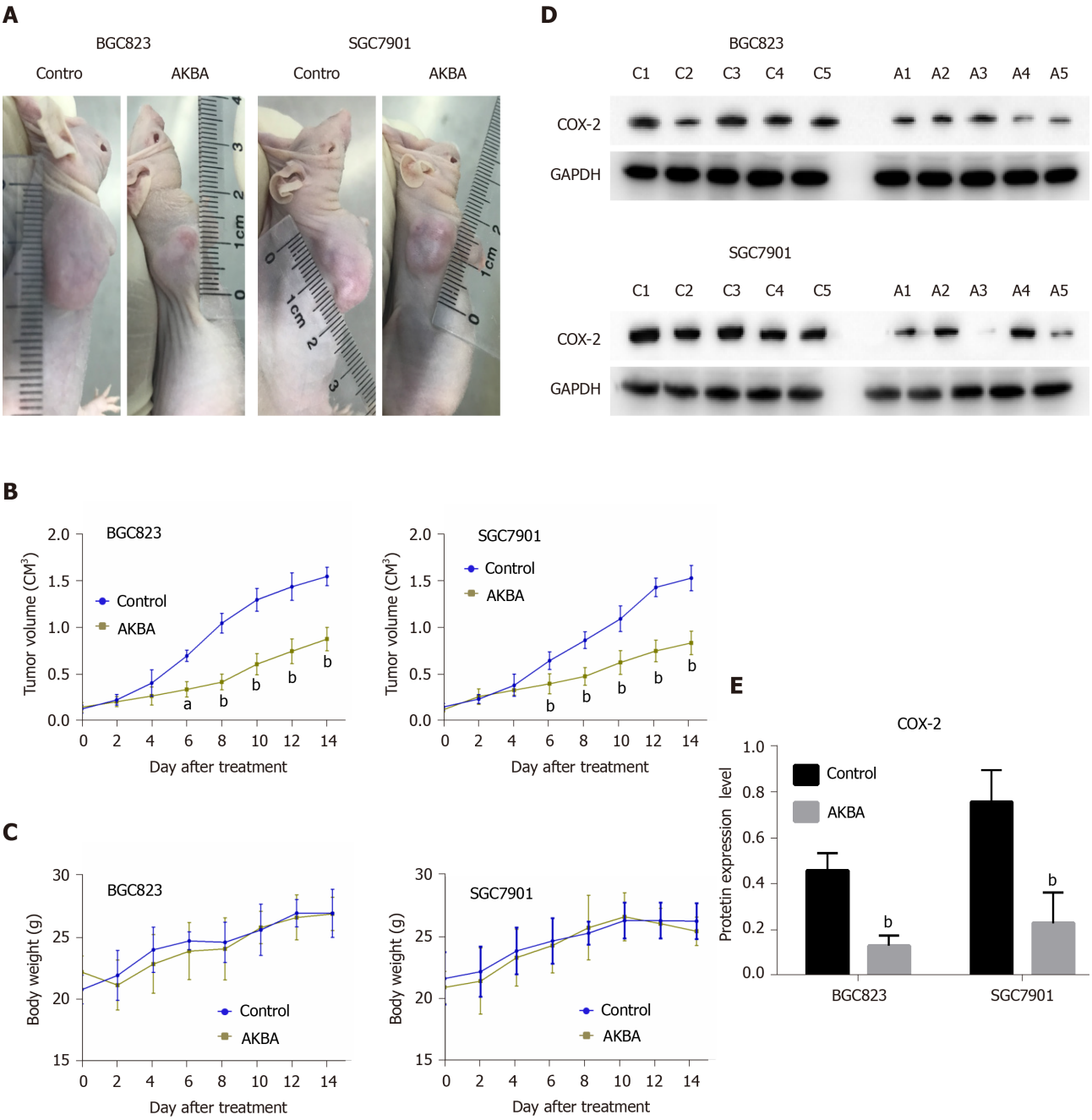

Figure 6 Acetyl-11-keto-β-boswellic acid inhibits gastric cancer tumor growth *in vivo*. Twenty nude mice were subcutaneously injected with BGC823 and SGC7901 cells. When tumor size was approximately 0.1 cm^3, the nude mice were randomly divided into different groups (n = 5) to receive different treatments. A: Acetyl-11-keto-β-boswellic acid (AKBA) inhibits gastric cancer xenograft tumor growth *in vivo*; B: Tumor volume was measured, and the tumor growth curves were plotted; C: Body weight change in the nude mice treated with saline or AKBA; D: Western blot analysis of cyclooxygenase (COX)-2 protein expression in gastric cancer tissues of control and AKBA treated nude mice (100 mg/kg/d); E: Histogram representing the relative expression of COX-2 compared with that of GAPDH. $^aP < 0.05$, $^bP < 0.01$ *vs* control (saline). AKBA: Acetyl-11-keto-β-boswellic acid.

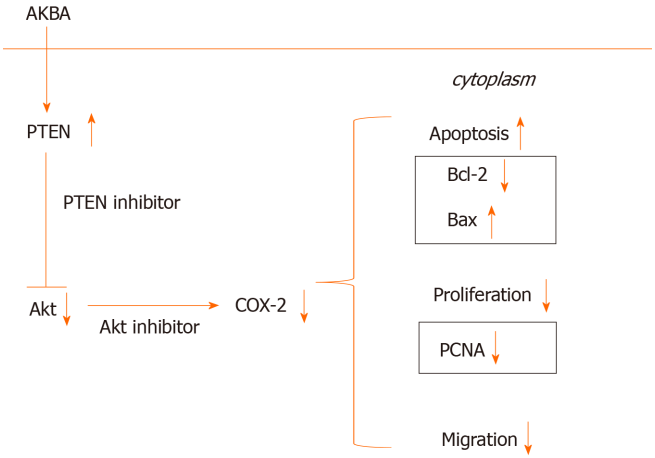

Figure 7 Hypothetical model of acetyl-11-keto-β-boswellic acid's inhibition of proliferation of gastric cancer cells and induction of apoptosis. AKBA: Acetyl-11-keto-β-boswellic acid; PTEN: Phosphatase and tensin homolog; COX-2: Cyclooxygenase-2; PCNA: Proliferating cell nuclear antigen.

ARTICLE HIGHLIGHTS

Research background

Gastric cancer is a digestive malignancy with high incidence and mortality globally. Despite therapeutic improvement, it remains a leading cause of cancer-associated deaths. Gastric cancer can be diagnosed early by endoscopy and treated by timely surgical excision, but the patient's long-term survival is still threatened by high metastasis, recurrence, and drug resistance. Active medicinal compounds from natural sources have provided alternative treatment options.

Research motivation

Gastric cancer is a global health threat. Cyclooxygenase (COX)-2 plays an important role in the carcinogenesis and progression of gastric cancer. The effects of acetyl-11-keto-β-boswellic acid (AKBA) on human gastric cancer remain unclear.

Research objectives

The objective of this study was to evaluate the anti-tumor effects and mechanisms of AKBA in human gastric cancer both *in vitro* and *in vivo*.

Research methods

Human gastric cancer cell lines BGC823 and SGC7901 were routinely cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum. Sub-confluent cell cultures were treated with AKBA at a final concentration of 10-50 μM for 24, 48, and 72 h, and the cell proliferation was determined using methyl thiazolyl tetrazolium colorimetric assay. Apoptosis was assessed *via* flow cytometry analysis. The wound healing assay was performed to evaluate the effects of AKBA on the migration of gastric cancer cells. The expression of proliferating cell nuclear antigen (PCNA), COX-2, Bcl-2, Bax, phosphatase and tensin homolog (PTEN), Akt, and phosphorylated Akt was detected by Western blot analysis. A xenograft nude mouse model of human gastric cancer was established to evaluate the anti-cancer effect of AKBA *in vivo*.

Research results

AKBA significantly inhibited cellular proliferation and migration and induced apoptosis *in vitro*, as well as inhibited tumor growth *in vivo*. Additionally, AKBA significantly inhibited the expression of PCNA, COX-2, Bcl-2, and phosphorylated Akt as well as increased PTEN and Bax expression in gastric cancer cells.

Research conclusions

AKBA could prevent the development of gastric cancer through the PTEN/Akt/COX-2 signaling pathway.

Research perspectives

COX-2 is involved in the carcinogenesis and development of gastric cancer. AKBA is a promising drug for gastric cancer therapy.

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ORIGINAL ARTICLE

Case Control Study

Endogenous motion of liver correlates to the severity of portal hypertension

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Author contributions: Kupcinskas L and Kupcinskas J designed the research; Gelman S, Zykus R and Kuliaviene I treated patients, collected material and clinical data from the patients; Sakalauskas A, Jurkonis R and Lukosevicius A analyzed the radiofrequency signals and developed the algorithm; Pranculis A performed hepatic vein catheterization and HVPG measurement; Gelman S performed ultrasound scanning, analysed the data and drafted the manuscript; Kupcinskas J approved the final version of the manuscript.

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Abstract

BACKGROUND

Degree of portal hypertension (PH) is the most important prognostic factor for the decompensation of liver cirrhosis and death, therefore adequate care for patients with liver cirrhosis requires timely detection and evaluation of the presence of clinically significant PH (CSPH) and severe PH (SPH). As the most accurate method for the assessment of PH is an invasive direct measurement of hepatic venous pressure gradient (HVPG), the search for non-invasive methods to diagnose these conditions is actively ongoing.

AIM

To evaluate the feasibility of parameters of endogenously induced displacements and strain of liver to assess degree of PH.

METHODS

Of 36 patients with liver cirrhosis and measured HVPG were included in the case-control study. Endogenous motion of the liver was characterized by derived parameters of region average tissue displacement signal (d_{antero}, dr_{etro}, d_{RMS}) and results of endogenous tissue strain imaging using specific radiofrequency signal processing algorithm. Average endogenous strain μ and standard deviation σ of

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Informed consent statement: All study participants provided informed written consent prior to study enrollment.

Conflict-of-interest statement: None declared.

Data sharing statement: Technical appendix and dataset available from the corresponding author at sigita.gelman@lsmuni.lt. Informed consent for data sharing was not obtained but the presented data are anonymized, and risk of identification is low.

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strain were assessed in the regions of interest (ROI) (1 cm × 1 cm and 2 cm × 2 cm in size) and different frequency subbands of endogenous motion (0-10 Hz and 10-20 Hz).

RESULTS

Four parameters showed statistically significant ($P < 0.05$) correlation with HVPG measurement. The strongest correlation was obtained for the standard deviation of strain (estimated at 0-10 Hz and 2 cm × 2 cm ROI size). Three parameters showed statistically significant differences between patient groups with CSPH, but only d_{retro} showed significant results in SPH analysis. According to ROC analysis area under the curve (AUC) of the $\sigma_{ROI[0...10Hz,\ 2\ cm \times 2\ cm]}$ parameter reached 0.71 ($P = 0.036$) for the diagnosis of CSPH; with a cut-off value of 1.28 μm/cm providing 73% sensitivity and 70% specificity. AUC for the diagnosis of CSPH for $\mu_{ROI[0...10Hz,\ 1\ cm \times 1\ cm]}$ was 0.78 ($P = 0.0024$); with a cut-off value of 3.92 μm/cm providing 73% sensitivity and 80% specificity. D_{retro} parameter had an AUC of 0.86 ($P = 0.0001$) for the diagnosis of CSPH and 0.84 ($P = 0.0001$) for the diagnosis of SPH. A cut-off value of -132.34 μm yielded 100% sensitivity for both conditions, whereas specificity was 80% and 72% for CSPH and SPH respectively.

CONCLUSION

The parameters of endogenously induced displacements and strain of the liver correlated with HVPG and might be used for non-invasive diagnosis of PH.

Key Words: Portal hypertension; Endogenous motion; Strain elastography; Hepatic venous pressure gradient; Radiofrequency parameters

Core Tip: In this study, we aimed to evaluate the feasibility of parameters of endogenously induced displacements and strain of the liver to assess the degree of portal hypertension. Endogenous motion of the liver was characterized by derived parameters of region average tissue displacement signal and results of endogenous tissue strain imaging using specific radiofrequency signal processing algorithm. Our proposed parameters correlated with hepatic venous pressure gradient and statistically significant differences were observed between patient groups with clinically significant and severe portal hypertension. To our knowledge it is the first study to evaluate prognostic potential of endogenous motion parameters to detect clinically significant and severe portal hypertension.

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INTRODUCTION

The burden of chronic liver diseases is growing and decompensated liver cirrhosis is responsible for a million of deaths per year worldwide[1]. The grade of portal hypertension (PH) expressed by hepatic venous pressure gradient (HVPG) is an important prognostic factor for the decompensation of liver cirrhosis and formation of life threatening complications, such as ascites, gastroesophageal varices and hepatic encephalopathy[2,3]. HVPG value greater than 5 mmHg is considered to be PH. Clinically significant PH (CSPH) is diagnosed when HVPG values exceed 10 mmHg and is associated with the formation of gastroesophageal varices, ascites and hepatorenal syndrome. When HVPG values exceed 12 mmHg severe PH (SPH) is diagnosed with higher risk of variceal bleeding and decompensation. Thus the evaluation of the presence of CSPH and SPH are of utmost importance[4,5].

Direct measurement of HVPG is regarded as the most accurate method for the assessment of PH, however this procedure is currently reserved for specialized centers, as it is invasive, costly and requires expertise[2,6].

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Ultrasound elastography is one of the most widely used non-invasive alternatives for the diagnosis of PH. The method is appealing due to its low cost, applicability and availability[4,7]. Several ultrasound elastography techniques have been developed: Strain imaging [strain elastography (SE), using internal/external compression stimuli, or acoustic radiation force impulse] and shear wave imaging [point shear wave elastography (pSWE), 2D-SWE and transient elastography (TE)][8,9]. Out of existing ultrasound elastography techniques TE, using Fibroscan (Echosens, France), has shown most promising results in diagnosing CSPH with area under the receiver operating characteristic curve (AUROC) of 0.90, sensitivity 87% and specificity 85%, whereas other shear wave techniques showed lower sensitivity in this field[10-12]. The readings of TE, pSWE and 2D-SWE are affected by active inflammation, cholestasis, fatty liver and biliary obstruction thus TE performance is not optimal in patients with obesity, ascites, narrow intercostal space. These limitations encourage the search for another alternative technique that could be applied in this diverse patient population.

SE is an ultrasound elastography technique widely used for the examination of musculoskeletal system[13], breast and thyroid pathologies[14], however it has been scarcely evaluated in the setting of liver cirrhosis and PH. SE measures axial displacement of tissue caused by manual compression or physiological shifts inside the body (cardiovascular pulsatility or breathing)[15,16]. Elasticity is displayed as color coded elastograms: Areas with lower strain are displayed in blue and areas with higher strain in red[11,15]. The drawback of this technique is that it measures the strain of tissues and the resulting elastogram is not quantifiable[16]. To obtain more accurate and reproducible results various semi-quantitative methods have been developed[15]. Different scores for semi-quantitative interpretation of SE have been proposed: The German Elasticity Score, the Japanese Elasticity Score, the LF Index, calculation of strain[9,17]. Other research groups develop and apply specific algorithms for quantitative evaluation of elasticity using a number of statistical parameters, derived from the distribution of recorded strains within a region of interest (ROI). The key parameters are: Mean strain, standard deviation of the mean; the percentage of blue area; complexity of the blue areas[18-23]. One advantage of this technique is the ability to evaluate the inhomogeneity of strain in larger areas of the liver[24,25]. Also, what is more important, SE readings are not affected by hepatic inflammation, jaundice, liver congestion, fatty degeneration, obesity, ascites or narrow intercostal spaces as is the case with other elastography modalities[15,26-28].

Studies concerning SE performance in PH are scarce. Ochi *et al*[29] reported that SE was useful for the diagnosis of PH in patients with non-alcoholic liver disease. Hirooka *et al*[30] evaluated SE performance in CSPH and SPH using elastic ratio. In their study AUROC for the diagnosis of CSPH was 0.83 and for the diagnosis of SPH – 0.78.

Our group has applied SE technique to assess the strain of liver tissue caused by endogenous motion of the beating heart and developed a specific radiofrequency (RF) signal analysis algorithm to calculate the parameters for quantification of strain in liver tissue. We have previously applied this RF ultrasound-based tissue strain imaging method to characterize tissue elasticity *in vitro*[31] and demonstrated the feasibility of this method in diagnosing liver fibrosis in patients with hepatitis C virus[32]. The aim of present study was to evaluate the ability and feasibility of endogenously induced displacements and strain on the liver to assess the degree of PH, using this specifically developed RF signal analysis algorithm.

MATERIALS AND METHODS

Patients

Patients with hepatitis C virus and/or alcohol induced liver cirrhosis, hospitalized for the HVPG measurement procedure to the Hospital of Lithuanian University of Health Sciences, Department of Gastroenterology were included in this study. Exclusion criteria were: Pre- or post-hepatic causes of PH, cardiovascular disease, current use of beta-blockers or other vasoactive drugs, presence of ascites. Demographic data, medical history, aethiology of liver disease and HVPG values were recorded. The diagnosis of liver cirrhosis was based on clinical, laboratory and radiologic data and/or histology.

The study was approved by Kaunas Region Biomedical Research Ethics Committee (2015-08-24, No. BE-2-28, Kaunas, Lithuania) and a written informed consent to participate in the study was acquired from all participants.

Ultrasound scanning

We collected data using ultrasound scanner Ultrasonix Sonix Touch (Analogic Ultrasound, Canada), which allows to collect raw RF signals of all scanning lines. The ultrasound scanning was performed on the day of HVPG measurement prior to the procedure. Scanning protocol and the main RF data acquisition parameters were described in previous paper[32].

HVPG measurement

Invasive HVPG measurement procedure was used to assess the degree of PH. The procedure was performed according to standard as described by previous authors[33]. Portal pressure was considered to be normal for HVPG values of 1-5 mmHg. When HVPG values exceeded 6 mmHg, PH was diagnosed. HVPG values of ≥ 10 mmHg represented clinically significant and ≥ 12 mmHg – SPH.

RF signal processing

Endogenous motion of the liver was characterized by derived parameters of region average tissue displacement signal and results of endogenous tissue strain imaging.

Displacement estimation

The displacements along the scanning beam line were estimated applying cross-correlation based technique. The signals were interpolated before calculation of cross-correlation. Length of the correlation window was set to 6 wavelengths and the overlap was 50%. The detailed description of the method could be found in previous publication[32]. The sequences of displacement images were obtained as a result of the displacement estimation stage.

Displacement parameters

The user predefined regions of interest (ROI) in all images of displacements were averaged in space, thus obtaining the region average liver tissue displacement signal. The average displacement signal could be expressed as follows:

Formula 1 where $p = 1…P$, P – number of displacement data points in a ROI of a subsector, $d_p[k]$ – displacement signal at p-th spatial position in a ROI, $k = 1…K$, K – acquisition instance (frame number).

Afterwards, the motion of the liver was characterized by three parameters of the region average tissue displacement signal (assessment example is presented in Figure 1A): (1) Maximal amplitude of motion towards the ultrasonic probe d_{antero}; (2) Maximal amplitude of motion backwards from the ultrasonic probe d_{retro}; and (3) Average level of motion by root-mean-square d_{RMS} value:

Formula 2 where $k = 1…K$, K – acquisition instance (frame number).

Strain parameters

The endogenous strain map was derived from the temporal sequence of the displacement images. The strain maps were obtained taking the gradient of integrated spectral coefficient. An example of derived strain map is presented in Figure 1B. The description of data processing methods used for parametric imaging of endogenous strain was presented in detail in previous study[32].

The local quantitative assessment of the strain was performed by analyzing the distribution of derived strain map values in user predefined rectangular ROI of constant size (see assessment example in Figure 1B). The investigation was performed in 2 frequency subbands (integration ranges for lower f_1 and f_2 frequencies 0-10 Hz and higher 10-20 Hz, respectively) and 2 sizes of ROI (1 cm × 1 cm and 2 cm × 2 cm). The ROI selection criteria were based on visual evaluation of B scans and the obtained endogenous strain maps. At first, the sequences of B mode images were carefully revised with the aim to identify appropriate regions. The regions that contained only liver parenchyma without clearly visible blood vessels in B scans were outlined by the observer in the spatially corresponding images of endogenous strain.

All 11 investigated parameters of endogenous motion of liver together with a description are provided in Table 1.

Statistical analysis

Statistical analysis was performed using SPSS 25.0 and Medcalc softwares. Kolmogorov–Smirnov test was used to check data normality. Descriptive statistics are provided as median and range for non-parametric data. Patients were divided into groups according to HVPG values: (1) Patients without CSPH (HVPG < 10 mmHg); (2)

Table 1 Investigated endogenous motion parameters

No.	Parameter	Description
1	d_{antero}	Maximal amplitude of endogenous displacements towards the probe, μm
2	d_{retro}	Maximal amplitude of the displacements backward, μm
3	d_{RMS}	Average level of motion, μm
4	$\mu_{ROI[0…10\ Hz,\ 2\ cm \times 2\ cm]}$	Average strain [estimated for the 0…10 Hz sub-band of endogenous motion in the 2 cm × 2 cm ROI], μm/cm
5	$\sigma_{ROI[0…10\ Hz,\ 2\ cm \times 2\ cm]}$	Standard deviation of strain [0…10 Hz, 2 cm × 2 cm], μm/cm
6	$\mu_{ROI[0…10\ Hz,\ 1\ cm \times 1\ cm]}$	Average strain [0…10 Hz, 1 cm × 1 cm], μm/cm
7	$\sigma_{ROI[0…10\ Hz,\ 1\ cm \times 1\ cm]}$	Standard deviation of strain [0…10 Hz, 1 cm × 1 cm], μm/cm
8	$\mu_{ROI[10…20\ Hz,\ 2\ cm \times 2\ cm]}$	Average strain [10…20 Hz, 2 cm × 2 cm], μm/cm
9	$\sigma_{ROI[10…20\ Hz,\ 2\ cm \times 2\ cm]}$	Standard deviation of strain [10…20 Hz, 2 cm × 2 cm], μm/cm
10	$\mu_{ROI[10…20\ Hz,\ 1\ cm \times 1\ cm]}$	Average strain [10…20 Hz, 1 cm × 1 cm], μm/cm
11	$\sigma_{ROI[10…20\ Hz,\ 1\ cm \times 1\ cm]}$	Standard deviation of strain [10…20 Hz, 1 cm × 1 cm], μm/cm

ROI: Region of interest; RMS: Root mean square.

$$\overline{d[k]} = \frac{1}{P} \cdot \sum_{p=1}^{P} d_p[k]$$

Formula 1

Patients with CSPH (HVPG ≥ 10 mmHg); (3) Patients without SPH (HVPG < 12 mmHg); and (4) Patients with SPH (HVPG ≥ 12 mmHg). Differences between the groups were evaluated using the Mann-Whitney's test. Correlations were performed using Spearman's correlation and expressed by Spearman's coefficient. ROC curves were created to assess the predictive values of RF parameters for the degree of PH. AUC, sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated. The cut-off value according to Youden's index was chosen for the analysis of RF parameters. Statistical significance was established at $P < 0.05$.

RESULTS

Of 36 patients were included in the study. Mean age (± SD) of the subjects was 54.25 ± 8.82; 58.3% were male. Hepatitis C induced liver cirrhosis was diagnosed in 63.9% of cases. The summary of the demographic data is presented in Table 2.

We determined the correlation between the investigated strain parameters and invasive HPVG measurement. Spearman's correlation coefficient (ρ) values and P value for the assessment of statistical significance are presented in Table 3. Four parameters showed statistically significant ($P < 0.05$) correlation with HVPG measurement. The strongest correlation was obtained for the standard deviation of strain (estimated at 0-10 Hz integration range and 2 cm × 2 cm ROI size). The directions of correlations met the expectation in almost all the cases. Negative correlation confirmed that the derived strain estimates decreased with an increment of HVPG.

In the next stage we investigated the ability of these four parameters to evaluate CSPH (HVPG ≥ 10 mmHg) and SPH (HVPG ≥ 12 mmHg). The results are presented in boxplots as median and interquartile ranges (Figure 2 and 3). Three parameters (No. 2, 5, 6; Table 1) showed statistically significant differences between the groups.

According to ROC analysis d_{retro} parameter had an AUC of 0.86 ($P = 0.0001$) for the diagnosis of CSPH and 0.84 ($P = 0.0001$) for the diagnosis of SPH. A cut-off value of -132.34 μm yielded 100% sensitivity for both conditions, whereas specificity was 80% and 72% for CSPH and SPH respectively (Figure 4). AUC of the $\sigma_{ROI[0…10Hz,\ 2\ cm \times 2\ cm]}$

Table 2 Demographic and clinical data of the patients

Variable	Characteristics (*n* = 36)
Sex (male/female; %)	58.3/41.7
Age (yr; SD)	54.25 (8.82)
Aethiology (% of patients)	
Alcohol cirrhosis	36.1
HCV cirrhosis	63.9
Child-Pugh score (A/B/C; % of patients)	58.3/36.2/5.6
HVPG (mmHg; SD)	14.3 (5.9)
HVPG 1-5 mmHg (% of patients)	2.8
HVPG 6-9 mmHg (% of patients)	25
CSPH; HVPG ≥ 10 mmHg (% of patients)	72.2
SPH; HVPG ≥ 12 mmHg (% of patients)	69.4

HCV: Hepatitis C virus; HVPG: Hepatic venous pressure gradient; CSPH: Clinically significant portal hypertension; SPH: Severe portal hypertension.

Table 3 Correlations between the investigated parameters and hepatic venous pressure gradient

No.	Parameter	Spearman's *ρ*	*P* value
1	d_{antero}	-0.31	0.07
2	d_{retro}	0.34	0.04
3	d_{RMS}	-0.33	0.05
4	$\mu_{ROI[0...10\ Hz,\ 2 \times 2\ cm]}$	-0.38	0.04
5	$\sigma_{ROI[0...10\ Hz,\ 2\ cm \times 2\ cm]}$	-0.42	0.01
6	$\mu_{ROI[0...10\ Hz,\ 1\ cm \times 1\ cm]}$	-0.38	0.02
7	$\sigma_{ROI[0...10\ Hz,\ 1\ cm \times 1\ cm]}$	-0.27	0.11
8	$\mu_{ROI[10...20\ Hz,\ 2\ cm \times 2\ cm]}$	-0.19	0.28
9	$\sigma_{ROI[10...20\ Hz,\ 2\ cm \times 2\ cm]}$	-0.14	0.43
10	$\mu_{ROI[10...20\ Hz,\ 1\ cm \times 1\ cm]}$	-0.16	0.34
11	$\sigma_{ROI[10...20\ Hz,\ 1\ cm \times 1\ cm]}$	-0.16	0.36

ROI: Region of interest; RMS: Root mean square.

parameter reached 0.71 (P = 0.036) for the diagnosis of CSPH; with a cut-off value of 1.28 μm/cm providing 73% sensitivity and 70% specificity. AUC for the diagnosis of CSPH for $\mu_{ROI[0...10Hz,\ 1\ cm \times 1\ cm]}$ was 0.78 (P = 0.0024); with a cut-off value of 3.92 μm/cm providing 73% sensitivity and 80% specificity (Figure 5). Data on specificity, sensitivity, PPV and NPV are presented in Table 4.

DISCUSSION

Diagnosis of PH is currently based on the invasive procedure requiring high expertise; therefore, the need for an accurate non-invasive diagnostic test is growing. In this study for the non-invasive diagnosis of PH we applied a RF ultrasound-based tissue strain imaging method. We have shown that four out of 11 investigated parameters significantly correlated with HVPG and predicted CSPH and SPH. The d_{retro} parameter was a strong predictor of CSPH and SPH with sensitivity and NPV of 100%. Other authors have described similar performance in non–invasive diagnosis of CSPH of TE[34], whereas pSWE and 2D SWE methods were less sensitive (AUC 0.85 and 0.81

Table 4 Diagnostic performance of parameters for the diagnosis of portal hypertension

Parameter	Value	Sensitivity	Specificity	PPV	NPV	*P* value
d_{retro}						
CSPH	132.34 μm	100	80	93	100	0.0001
SPH		100	72	89	100	0.0001
$\sigma_{ROI[0…10\ Hz,\ 2\ cm \times 2\ cm]}$						
CSPH	1.28 μm/cm	73	70	86	50	0.036
$\mu_{ROI[0…10\ Hz,\ 1\ cm \times 1\ cm]}$						
CSPH	3.92 μm/cm	73	80	90	53	0.0024

CSPH: Clinically significant portal hypertension; SPH: Severe portal hypertension; PPV: Positive predictive value; NPV: Negative predictive value; ROI: Region of interest.

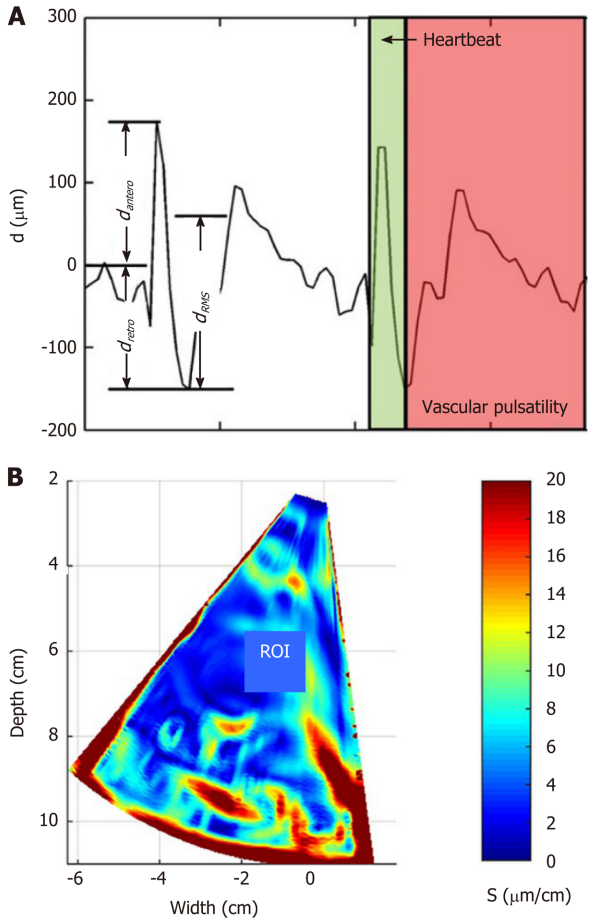

Figure 1 Example illustrating assessment of the endogenous liver displacements and strain. A: region averaged tissue displacement signal obtained in the subsector at time interval (2…3.8) s (three parameters of region averaged displacement signal d_{RMS}, d_{antero} and d_{retro} were evaluated in this study); B: the obtained strain map [amplitude coded in (μm/cm)] together with regions of interest (red rectangle, size 1 cm × 1 cm) used for the local assessment of endogenous strain. ROI: Regions of interest.

respectively, sensitivity 71% and 85% respectively[35,36]).

The presented technique has certain limitations. It does not allow the evaluation of strain in real-time differently from commercial SE techniques and the strain parameters are calculated retrospectively, using recorded RF data. The other limitation of the approach is the detection of displacements field only in the direction of ultrasound wave propagation. Our method reveals the estimates of the displacements and strain only in single projection of endogenous motion, which is actually three-dimensional. The second projection of motion could be estimated by updating the

$$d_{RMS} = \sqrt{\frac{1}{K} \cdot \sum_{k=1}^{K} \overline{d^2[k]}}$$

Formula 2

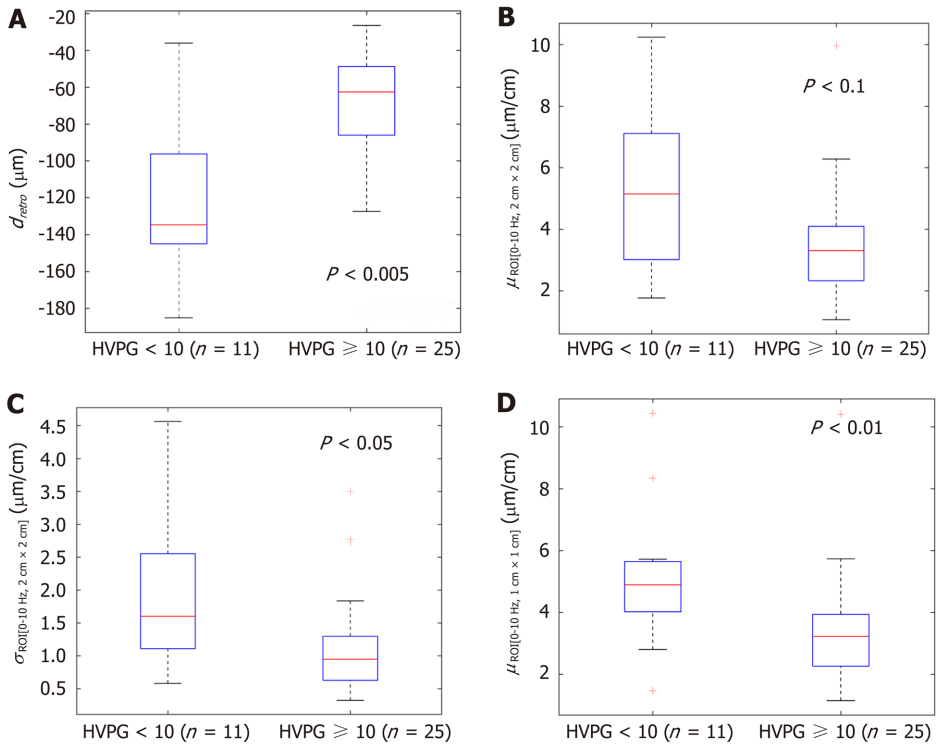

Figure 2 The boxplots and *P* values representing the derived parameters of endogenous displacements and strain in patients with and without clinically significant portal hypertension (≥ 10 mmHg). A: d_{retro}; B: $\mu_{ROI[0...10Hz,\ 2\ cm \times 2\ cm]}$; C: $\sigma_{ROI[0...10Hz,\ 2\ cm \times 2\ cm]}$; D: $\mu_{ROI[0...10Hz,\ 1\ cm \times 1\ cm]}$. HVPG: Hepatic venous pressure gradient.

algorithm for two-dimensional detection, but sector shaped scan requires complicated computations.

In this method we have used an undefined endogenous source for the tissue displacement excitation, which might influence quantified evaluation of liver elasticity. The endogenous motion also has a complex pattern which is composed from the very slow waves, induced by respiratory activity, a bit faster vascular pulsatility and relatively fast mechanical strain induced by the heartbeat and motion artefacts. However, the exact definition of motion inducing sources is beyond this study and will be investigated in future research. Other technical limitations of the method were disclosed in the previous study[32]. Also, despite the promising results, this was a pilot study and our results could be biased by the small size of our sample.

CONCLUSION

In conclusion, the parameters of endogenously induced displacements and strain of the liver significantly correlated with HVPG and could be a potential diagnostic tool for the non-invasive diagnosis of PH. A proposed method is potentially suitable for other applications of non-invasive diagnostics.

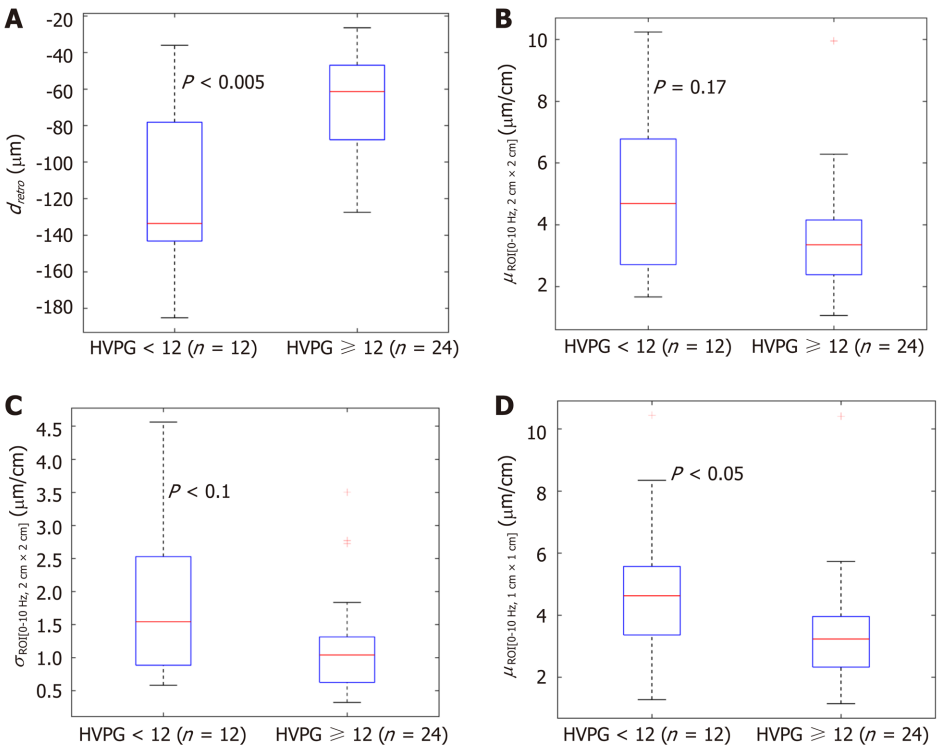

Figure 3 The boxplots and *P* values representing the derived parameters of endogenous displacements and strain in patients with and without severe portal hypertension (≥ 12 mmHg). A: *d*retro; B: $\mu_{ROI[0...10Hz,\ 2\ cm \times 2\ cm]}$; C: $\sigma_{ROI[0...10Hz,\ 2\ cm \times 2\ cm]}$; D: $\mu_{ROI[0...10Hz,\ 1\ cm \times 1\ cm]}$. HVPG: Hepatic venous pressure gradient.

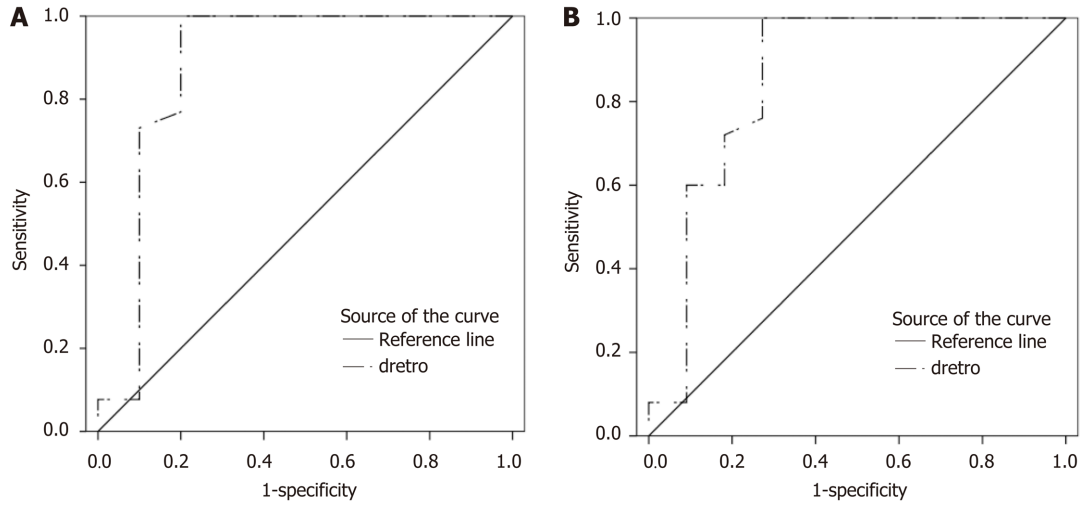

Figure 4 Receiver operating characteristic curves of d_{retro} parameter for the diagnosis of portal hypertension. A: Receiver operating characteristic (ROC) curve for clinically significant portal hypertension [hepatic venous pressure gradient (HVPG) ≥ 10 mmHg]; B: ROC curve for severe portal hypertension (HVPG ≥ 12 mmHg).

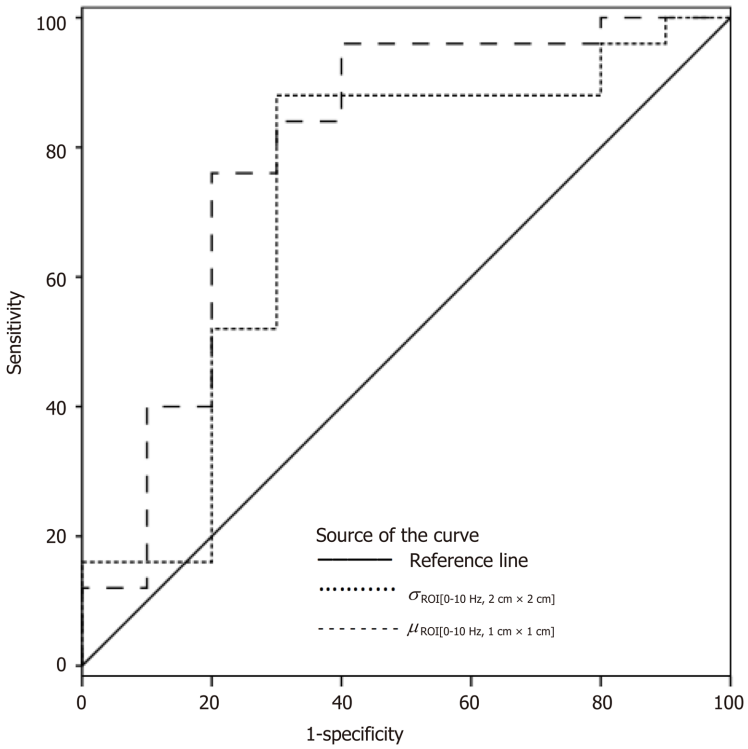

Figure 5 Receiver operating characteristic curves of $\sigma_{ROI[0–10Hz,\ 2\ cm \times 2\ cm]}$ and $\mu_{ROI[0–10Hz,\ 1\ cm \times 1\ cm]}$ parameters for the diagnosis of clinically significant portal hypertension (hepatic venous pressure gradient ≥ 10 mmHg).

ARTICLE HIGHLIGHTS

Research background

Degree of portal hypertension (PH) is the most important prognostic factor for the decompensation of liver cirrhosis and death, therefore adequate care for patients with liver cirrhosis requires timely detection and evaluation of the presence of clinically significant PH (CSPH) and severe PH (SPH).

Research motivation

As the most accurate method for the assessment of PH is an invasive direct measurement of hepatic venous pressure gradient (HVPG), the search for non-invasive methods to diagnose these conditions is actively ongoing. Ultrasound elastography is one of the most widely used non-invasive alternatives for the diagnosis of PH. Strain elastography (SE) is an ultrasound elastography technique widely used for the examination of musculoskeletal system, breast and thyroid pathologies, however it has been scarcely evaluated in the setting of liver cirrhosis and PH. It is an appealing alternative, as it is considered that SE readings are not affected by hepatic inflammation, jaundice, liver congestion, fatty degeneration, obesity, ascites or narrow intercostal spaces as is the case with other elastography modalities.

Research objectives

Our group has applied SE technique to assess the strain of liver tissue caused by endogenous motion of the beating heart and developed a specific radiofrequency (RF) signal analysis algorithm to calculate the parameters for quantification of strain in liver tissue. The aim of present study was to evaluate the ability and feasibility of endogenously induced displacements and strain on the liver to assess the degree of PH, using this specifically developed RF signal analysis algorithm.

Research methods

Of 36 patients with liver cirrhosis and measured HVPG were included in the case-control study. Endogenous motion of the liver was characterized by derived parameters of region average tissue displacement signal (d_{antero}, d_{retro}, d_{RMS}) and results of endogenous tissue strain imaging using specific radiofrequency signal processing algorithm. Average endogenous strain μ and standard deviation σ of strain were

assessed in the regions of interest (ROI) (1 cm × 1 cm and 2 cm × 2 cm in size) and different frequency subbands of endogenous motion (0-10 Hz and 10-20 Hz).

Research results

Four parameters showed statistically significant ($P < 0.05$) correlation with HVPG measurement. The strongest correlation was obtained for the standard deviation of strain (estimated at 0-10 Hz and 2 cm × 2 cm ROI size). Three parameters showed statistically significant differences between patient groups with CSPH, but only d_{retro} showed significant results in SPH analysis. According to ROC analysis area under the curve (AUC) of the $\sigma_{ROI[0...10Hz,\ 2\ cm\ \times\ 2\ cm]}$ parameter reached 0.71 ($P = 0.036$) for the diagnosis of CSPH; with a cut-off value of 1.28 μm/cm providing 73% sensitivity and 70% specificity. AUC for the diagnosis of CSPH for $\mu_{ROI[0...10Hz,\ 1\ cm\ \times\ 1\ cm]}$ was 0.78 ($P = 0.0024$); with a cut-off value of 3.92 μm/cm providing 73% sensitivity and 80% specificity. D_{retro} parameter had an AUC of 0.86 ($P = 0.0001$) for the diagnosis of CSPH and 0.84 ($P = 0.0001$) for the diagnosis of SPH. A cut-off value of -132.34 μm yielded 100% sensitivity for both conditions, whereas specificity was 80% and 72% for CSPH and SPH respectively.

Research conclusions

The parameters of endogenously induced displacements and strain of the liver significantly correlated with HVPG. This shows that parameters of endogenous motion could be a potential diagnostic tool for the non-invasive diagnosis of PH. A proposed method is potentially suitable for other applications of non-invasive diagnostics.

Research perspectives

The presented technique has certain limitations. It does not allow the evaluation of strain in real-time differently from commercial SE techniques and the strain parameters are calculated retrospectively, using recorded RF data. The other limitation of the approach is the detection of displacements field only in the direction of ultrasound wave propagation. In this method we have used an undefined endogenous source for the tissue displacement excitation, which might influence quantified evaluation of liver elasticity. Also despite the promising results, this was a pilot study and our results could be biased by the small size of our sample. We are conducting further research of this method, which will include larger groups of patients, also allowing subgroup analysis of patients with ascites and obesity.

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ORIGINAL ARTICLE

Case Control Study

Longitudinal decrease in platelet counts as a surrogate marker of liver fibrosis

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Author contributions: Gotlieb N collected the data and wrote the manuscript; Schwartz N analyzed the results and performed the statistical analysis; Zelber-Sagi S, Chodick G and Shalev V were involved in critical revision of the paper; Shibolet O conceived the idea of the paper and was involved in writing and critical revision of the paper.

Institutional review board statement: The study was reviewed and approved by institutional review board (IRB) at the Maccabi Health Services (MHS).

Informed consent statement: Since this is a retrospective study in which anonymized administrative data from electronic medical records was retrieved, exemption from informed consent was granted by the IRB committee.

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Abstract

BACKGROUND

Liver cirrhosis is a significant source of morbidity and mortality worldwide. The disease is usually indolent and asymptomatic early in its course while many cirrhotic patients are diagnosed late when severe complications occur. A major challenge is to diagnose advanced fibrosis as early as possible, using simple and non-invasive diagnostics tools. Thrombocytopenia represents advanced fibrosis and portal hypertension (HTN) and most non-invasive scores that predict liver fibrosis incorporate platelets as a strong risk factor. However, little is known about the association between longitudinal changes in platelet counts (PTC), when still within the normal range, and the risk of cirrhosis.

AIM

To explore whether platelet counts trajectories over time, can predict advanced liver fibrosis across the different etiologies of liver diseases.

METHODS

A nested case-control study utilizing a large computerized database. Cirrhosis cases (n = 5258) were compared to controls (n = 15744) matched for age and sex at a ratio of 1:3. All participants had multiple laboratory measurements prior to enrollment. We calculated the trends of PTC, liver enzymes, bilirubin, international normalized ratio, albumin and fibrosis scores (fibrosis-4 and

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aspartate transaminase-to-platelet ratio index) throughout the preceding 20 years prior to cirrhosis diagnosis compared to healthy controls. The association between PTC, cirrhosis complications and fibrosis scores prior to cirrhosis diagnosis was investigated.

RESULTS

The mean age in both groups was 56 (SD 15.8). Cirrhotic patients were more likely to be smokers, diabetic with chronic kidney disease and had a higher prevalence of HTN. The leading cirrhosis etiologies were viral, alcoholic and fatty liver disease. The mean PTC decreased from 240000/μL to 190000/μL up to 15 years prior to cirrhosis diagnosis compared to controls who's PTC remained stable around the values of 240000/μL. This trend was consistent regardless of sex, cirrhosis etiology and was more pronounced in patients who developed varices and ascites. Compared to controls whose values remained in the normal range, in the cirrhosis group aspartate aminotransferase and alanine aminotransferase, increased from 40 U/L to 75 U/L and FIB-4 increased gradually from 1.3 to 3 prior to cirrhosis diagnosis. In multivariable regression analysis, a decrease of 50 units in PTC was associated with 1.3 times odds of cirrhosis (95%CI 1.25-1.35).

CONCLUSION

In the preceding years before the diagnosis of cirrhosis, there is a progressive decline in PTC, within the normal range, matched to a gradual increase in fibrosis scores.

Key Words: Cirrhosis; Platelets; Count; Trend; Prediction; Range

Core tip: Cirrhosis is usually asymptomatic thus often diagnosed late when complications occur. Most non-invasive hepatic fibrosis scores (fibrosis-4, aspartate transaminase-to-platelet ratio index) incorporate platelets as a strong risk factor. However, the association between platelets average trends within the normal range and the risk of cirrhosis development is unknown. We found that up to 15 years before the diagnosis of cirrhosis, a progressive decline in platelet counts occurs, within the normal limits along with a gradual incline in FIB-4. The decline in platelet counts may alert of an early liver disease and may enable early therapeutic and preventive interventions before complications occur.

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INTRODUCTION

Liver cirrhosis is an important public health concern and a significant cause of morbidity and mortality worldwide. The global prevalence of cirrhosis ranges from 4.5% to 9.5% of the general population and is likely to increase due to the aging of hepatitis C virus (HCV) patients and rise in non-alcoholic fatty liver disease (NAFLD)[1,2]. In 2017, Cirrhosis caused more than 1.32 million deaths globally, compared with less than 899000 deaths in 1990. Most of the cases were secondary to decompensated liver disease[3]. Chronic liver disease (CLD) is usually indolent and asymptomatic early in its course, thus many cirrhotic patients are diagnosed late, when manifestations of portal hypertension (HTN) such as variceal bleeding, ascites or hepatocellular carcinoma (HCC) appear. Early diagnosis of cirrhosis is important in order to enroll patients into HCC surveillance programs and offer therapeutic interventions to halt or reverse disease progression. Nearly 1.5% of patients with cirrhosis remain undiagnosed throughout life, therefore, better diagnostics tools using laboratory and imaging modalities are needed[4].

Patients with advanced liver disease and cirrhosis may present changes in

laboratory values such as thrombocytopenia, hypoalbuminemia, abnormal clotting function, anemia, and changes in hepatocellular and cholestatic liver enzymes. Thrombocytopenia (platelet count < 150000/μL) is one of the most common abnormalities in patients with cirrhosis, seen in up to 78% of cirrhotic patients[5]. Thrombocytopenia carries important prognostic information in terms of the presence of cirrhosis, portal hypertensive complications, hepatocellular carcinoma, post-liver resection and the post-transplant course[6]. Indeed, there is a correlation between the degree of thrombocytopenia and the stage and severity of liver disease; severe thrombocytopenia (< 50000/μL) is a poor prognostic factor associated with significant morbidity, indicating an advanced liver disease with established portal HTN[7,8]. This strong association has been corroborated by a study indicating that liver diseases is the underlying cause of thrombocytopenia in 58% of outpatients from all hospital departments[9].

The pathogenesis of thrombocytopenia in CLD and liver cirrhosis is multifactorial. Possible causes include splenic sequestration of platelets, suppression of platelet production in the bone marrow, decreased thrombopoetin production in the liver and an autoimmune mediated destruction[5]. Additionally, platelets actively participate in pathophysiologic processes in the liver, resulting in fibrosis and cirrhosis; previous studies including animal models showed that platelets have a major role in liver inflammation *via* interactions with the hepatic sinusoidal endothelium and myeloid cells, inducing diverse hepatic processes ranging from liver repair and regeneration to necroinflammation and fibrosis[10,11]. Additionally, studies hypothesized that circulating platelet-neutrophil aggregates can induce neutrophil activation, thus driving end organ damage in patients with cirrhosis. Indeed, various liver diseases are associated with neutrophil recruitment; these include cholestatic liver injury, alcoholic hepatitis, drugs and chemical-induced injury[12].

While the association between thrombocytopenia and cirrhosis is well-established, little is known about the association between subtle changes in platelet counts over time and the long-term risk of cirrhosis development. Few previous studies have shown that platelet counts may start to fall earlier in the course of NAFLD and HCV induced liver diseases[13,14]. Additionally, platelet counts have been incorporated into non-invasive tools for the diagnosis of liver fibrosis and cirrhosis. Among others, these are the aspartate aminotransferase-to-platelet ratio index (APRI), fibrosis-4 (FIB-4) score and NAFLD fibrosis score which are used to assess the presence of liver fibrosis[15,16]. However, the platelet values in the aforementioned scores and studies were taken as a single value at a single time point. No study has tested the association between platelet trends within the normal range and cirrhosis incidence. Current computerized systems allow the collection of big data sets and enable the detection of subtle platelet changes, decades prior to the diagnosis of liver cirrhosis. Subtle trends in laboratory results are now being incorporated in machine learning algorithms which utilize artificial intelligence to generate predictive models more effectively than conventional methods, through detection of hidden patterns within large data sets.

In this study we aimed to explore whether platelet counts trajectories over time can advance the diagnosis of early liver disease and its predictive ability across the different etiologies of cirrhosis, in parallel to different fibrosis scores. In addition, we aimed to test the association between platelets decline and portal HTN complications (variceal bleeding, ascites, hepatic encephalopathy, HCC) among cirrhotic patients.

MATERIALS AND METHODS

Setting

A nested case-control study with diagnosed cirrhosis patients and matched controls, utilizing the Maccabi Health Services (MHS) database was performed. MHS is a 2.3-million-member state-mandated health services organization, representing 25% of the local population of Israel[17]. MHS's data are automatically collected and include information regarding all diagnoses, comorbidities, hospitalizations, emergency department visits, physician visits, outpatient specialist visits, purchase of medications, laboratory tests and radiologic imaging results. MHS's database was established and collects data from 1998, with a 99% members' retention rate which enables a unique opportunity to assess the long-term trends in laboratory results. All biochemical assessments are performed by a single laboratory that maintains a quality management system, as required and using the same standard laboratory methods[18]. The data are automatically and continuously updated, and are not dependent on active reporting by physicians.

Study population

Cases included all cirrhotic patients aged 18 to 80 years diagnosed between 2001 and 2018 using the International Classification of Diseases, 9th Revision (ICD-9) codes (Supplementary table 1). The first diagnosis was defined as the index date.

Controls were hepatic disease-free MHS members, matched for age, sex and birth country at a ratio of 1:3. Sampling date in the control group was matched to the cirrhosis diagnosis date. All study patients were required to have at least three PTC measurements prior to index date.

Patients with known etiologies for thrombocytopenia other than cirrhosis (various diseases and medications), as indicated in the medical record, were excluded from analysis (ICD-9 codes in Supplementary Tables 2 and 3).

Clinical data

Cirrhosis etiology (viral, autoimmune/cholestatic, NAFLD) as well as data regarding the complications of cirrhosis and portal HTN (hepatocellular carcinoma, ascites, varices, hepatic encephalopathy, splenomegaly) were all based on ICD-9 codes (Supplementary Table 4). We calculated the longitudinal trends of PTC as well the following laboratory parameters throughout the preceding 20 years prior to cirrhosis diagnosis compared to healthy controls: Complete blood count, bilirubin (total), liver enzymes [aspartate aminotransferase (AST), alanine transaminase (ALT), gamma-glutamyl transferase (GGT) and alkaline phosphatase], coagulation tests [prothrombin time (PT/INR), aPTT] and albumin were recorded (normal ranges are presented in Supplementary Table 5). APRI and FIB-4 were calculated for each patient according to the previously described formulas[15].

Ethical consideration

The study was approved by the MHS institutional review board (IRB). Since this is a retrospective study in which we used coded (anonymized) administrative data from electronic medical records, exemption from informed consent was granted by the IRB committee.

Statistical analysis

The statistical analyses were performed using SAS 9.4 software (SAS Institute Inc., Cary, NC, United States). Significance was set at $P < 0.05$. Categorical variables are presented using frequencies and percent. Continuous variables are presented using mean (standard deviation) [median, interquartile range]. The non-parametric locally weighted scatterplot smoothing was used for the presentation of the PTC (as well as other laboratory measurements) throughout 15 years period. For the cirrhosis group, the measurements were prior to the cirrhosis diagnosis and for the control group, the measurements were prior to the sampling year of each individual. Multivariable logistic regression was performed using PROC GENMOD utilizing general estimation equation methodology for correlated data (*i.e.*, several platelets measurements for each subject). The model included the platelets measurements, as well as the time gaps (in years) of each measurement from the diagnosis/sample year for the cirrhosis and control respectively. The number of measurements was also included in the model. Adjusted odds ratio as well as 95%CI were used to display the association between the study groups and the potential risk factors.

RESULTS

Characteristics of the study population

Characteristics of study population are presented in Table 1. The mean age in both groups was 56 (SD 15.8) and 54% were females. Most patients (25.7%) were diagnosed with cirrhosis in the years 2009-2012 while the least (14%) were diagnosed earlier between 2001 to 2004. Co-morbid conditions are presented in Table 2. Cases were more likely to be smokers (OR = 1.5; 95%CI: 1.39-1.6) as well as to be diagnosed with diabetes (OR = 1.17; 95%CI: 1.02-1.33), chronic kidney disease (CKD, OR = 1.24; 95%CI: 1.09-1.4) and tended to have higher prevalence of HTN (OR = 1.15; 95%CI: 0.96-1.37).

Cirrhosis etiology and complications

Of the cases with known etiology (n = 2058), the most common etiology for liver disease was viral infection (48%) followed by alcoholic liver disease (ALD, 24%) and NAFLD (20%). A total of 2768 cases had complications of liver cirrhosis, including

Table 1 Demographic characteristic of cases with cirrhosis and controls, *n* (%)

	Cirrhosis (*n* = 5258)	Control (*n* = 15774)
Age (yr)	55.91 (15.83), (56, 17-99)	56.04 (16.43), (57, 17-108)
17-30	338 (6.43)	1014 (6.43)
30-40	633 (12.04)	1899 (12.04)
40-50	929 (17.67)	2787 (17.67)
50-60	1215 (23.11)	3645 (23.11)
60-70	1124 (21.38)	3372 (21.38)
70-80	731 (13.9)	2193 (13.9)
80+	288 (5.48)	864 (5.48)
Gender		
Female	2866 (54.51)	8598 (54.51)
Male	2392 (45.49)	7176 (45.49)
Country of birth		
Africa and Middle East	273 (5.19)	819 (5.19)
America	73 (1.39)	219 (1.39)
Asia	19 (0.36)	57 (0.36)
Europe	2334 (44.39)	7002 (44.39)
Israel	2425 (46.12)	7275 (46.12)
Data N/A	134 (2.55)	402 (2.55)
Diagnosis/sampling year		
2001-2004	732 (13.92)	2196 (13.92)
2005-2008	1263 (24.02)	3789 (24.02)
2009-2012	1351 (25.69)	4053 (25.69)
2013-2015	1068 (20.31)	3204 (20.31)
2016-2018	844 (16.05)	2532 (16.05)

splenomegaly (14.5%), varices (10.5%), ascites (8.5%) and hepatic encephalopathy (5.6%). Portal vein thrombosis was documented in 1.3% of cases and 10 patients (0.19%) had HCC (Table 3).

Platelets trends along 15 years prior to cirrhosis diagnosis and comparison to controls

In both groups, the mean time gap between the first PTC and the diagnosis/sampling year was similar (7.57-7.68 years) and the mean number of platelets measurements increased gradually from 2.5 (SD 2.1) to 6.4 (SD 5.7) close to the diagnosis date.

The platelets trends along the study years (total 250646 platelets measurements) stratified by the study groups are presented in Figure 1. The mean PTC in the cirrhosis group decreased from 240000/μL starting 15 years prior to cirrhosis diagnosis to approximately 190000/μL close to the diagnosis date. In the control group, the PTC remained stable throughout the years (240-250000/μL). In addition, for each subject in both groups, the mean PTC was calculated per year; the difference in mean PTC per year between groups is presented in Supplementary Table 6.

Males had lower baseline PTC in both groups (Supplementary figure 1). In the cirrhosis group, males had a mean PTC of 210000 decreasing to 170000/μL *vs* females, with a mean number of 250000 decreasing to 215000/μL. In the control group, the same pattern was observed: males had a mean PTC of 225-230000/μL compared to females with ranges of 250000/μL. However, despite the sex differences, the trend of gradual decrease in PTC prior the diagnosis of cirrhosis was seen in both sexes. Additional sub-grouping was performed in order to assess whether age had a modifying effect within each sex. Among younger patients (17-40), PTC was constant

Table 2 Comparison between the number of platelets measurements and co-morbidities between cases with cirrhosis and controls, *n* (%)

	Cirrhosis (*n* = 5258)	Control (*n* = 15774)	OR	95%CI	
				Low	Up
Platelets measurements	15.59 (14.36) (11, 3-264)	10.69 (10.33) (7, 3-152)	1.033	1.031	1.036
Platelets measurements: 3-10	2510 (47.74)	10482 (66.45)	1		
11-20	1409 (26.8)	3395 (21.52)	1.73	1.61	1.87
21+	1339 (25.47)	1897 (12.03)	2.95	2.72	3.20
Diabetes	326 (6.2)	847 (5.37)	1.17	1.02	1.33
HTN	170 (3.23)	447 (2.83)	1.15	0.96	1.37
CKD	390 (7.42)	961 (6.01)	1.24	1.09	1.4
Dyslipidemia	915 (17.4)	2843 (18.02)	0.96	0.88	1.04
Smoking	1563 (29.73)	3470 (22)	1.5	1.398	1.609

HTN: Hypertension; CKD: Chronic kidney disease.

Table 3 Distribution of cirrhosis etiology and complications among cirrhotic patients (*n* = 5258)

	Frequency	Percent
Etiology		
ALD	488	9.28
Viral	987	18.77
Autoimmune	159	3.02
Wilson	9	0.17
NAFLD	413	7.85
Complications		
Ascites	450	8.56
Varices	551	10.48
HE	294	5.59
HCC	10	0.19
SBP	118	2.24
Portal HTN	511	9.72
Splenomgally	764	14.53
PVT	70	1.33

ALD: Alcoholic liver disease; NAFLD: Non-alcoholic fatty liver disease; HE: Hepatic encephalopathy; HCC: Hepatocellular carcinoma; SBP: Spontaneous bacterial peritonitis; HTN: Hypertension; PVT: Portal vein thrombosis.

in both males and females throughout the years, while among older patients, a trend of gradual decrease in PTC was seen in both sexes prior to cirrhosis diagnosis.

In contrast, there was no significant change in hemoglobin levels in both groups (range of 12.8-13.4 g/dL). But, there was a steep decrease in white blood cell (WBC) counts prior the diagnosis of cirrhosis compared to controls, which had an increase of these values (Supplementary figures 2 and 3).

Platelets trends among cirrhosis patients by etiology and complications

In the cirrhosis group, the trend in PTC was calculated and compared among the most common etiologies of liver cirrhosis in the cohort (viral and ALD). There was a

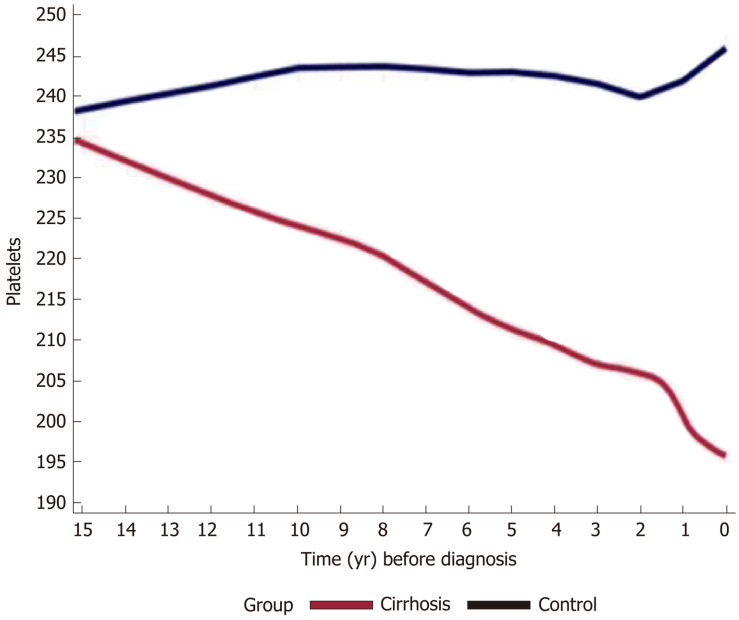

Figure 1 Trends in platelet counts across 15 years prior to cirrhosis diagnosis among cases and controls (*n* total = 21032, 250646 platelets measurements). Done with locally weighted scatterplot smoothing trend. The mean platelet counts in the cirrhosis group decreased from 240000/μL to 190000/μL, starting 15 years prior to cirrhosis diagnosis, compared to stable values in the control group.

gradual decrease in PTC prior to cirrhosis diagnosis, within the normal ranges in both etiologies. However, cirrhotic patients with ALD had lower mean platelets levels compared to those with viral liver disease, starting 15 years prior to cirrhosis diagnosis (230-180000/μL *vs* 200-170000/μL) (Supplementary figure 4A and B). Stratification of cases by complications of cirrhosis and portal HTN revealed a steeper decrease in PTC in cirrhotic patients who had esophageal varices, ascites and hepatosplenomegaly compared to cirrhotic patients with no such complications (Figure 2A and B).

Trends of liver enzymes and other laboratory markers of liver function

The trends of liver enzymes during the years prior to the diagnosis of cirrhosis (or sampling year for the control group) were calculated (Supplementary Figures 5-8). Compared to controls, whose enzymes levels remained stable and within the normal range, there was a gradual increase in both ALT and AST in cirrhotic patients during the 15 years preceding the diagnosis of liver cirrhosis, whose mean levels were both above the normal range: ALT increased from 50 U/L 15 years prior diagnosis to 75 U/L close to the diagnosis date; AST increased from 40 U/L to 70 U/L close to the diagnosis date.

Similarly, a gradual increase in both cholestatic enzymes could be seen during the years prior to the diagnosis of cirrhosis: Alkaline phosphatase increased from 75 U/L 15 years prior diagnosis to 135 U/L close to the diagnosis dates; GGT increased from 60 U/L to 200 U/L close to the diagnosis date. As for the control group, there was a gradual mild increase in both enzymes over the years.

Regarding markers of the synthetic functions of the liver, a gradual increase in bilirubin levels occurred within the normal range, in the cirrhosis group compared to controls. Albumin levels decreased in both groups, but remained within the normal range (Supplementary Figure 9).

Trends in fibrosis scores

We calculated FIB-4 and APRI for both cirrhotics and controls. In the preceding years before cirrhosis diagnosis, FIB-4 and APRI increased gradually, ranging from 1.3 to 3 and 0.48 to 0.93 respectively, compared to controls whose scores either increased minimally or remained stable respectively throughout the years (Figure 3A and B).

Association between PTC and cirrhosis in multivariate analysis

Since cases were matched with controls by age, gender, birth country and time period (*i.e.* cirrhosis diagnosis year and the sampling year for the control group), these factors were adjusted by selection. After adjusting for the platelet's measurements time points

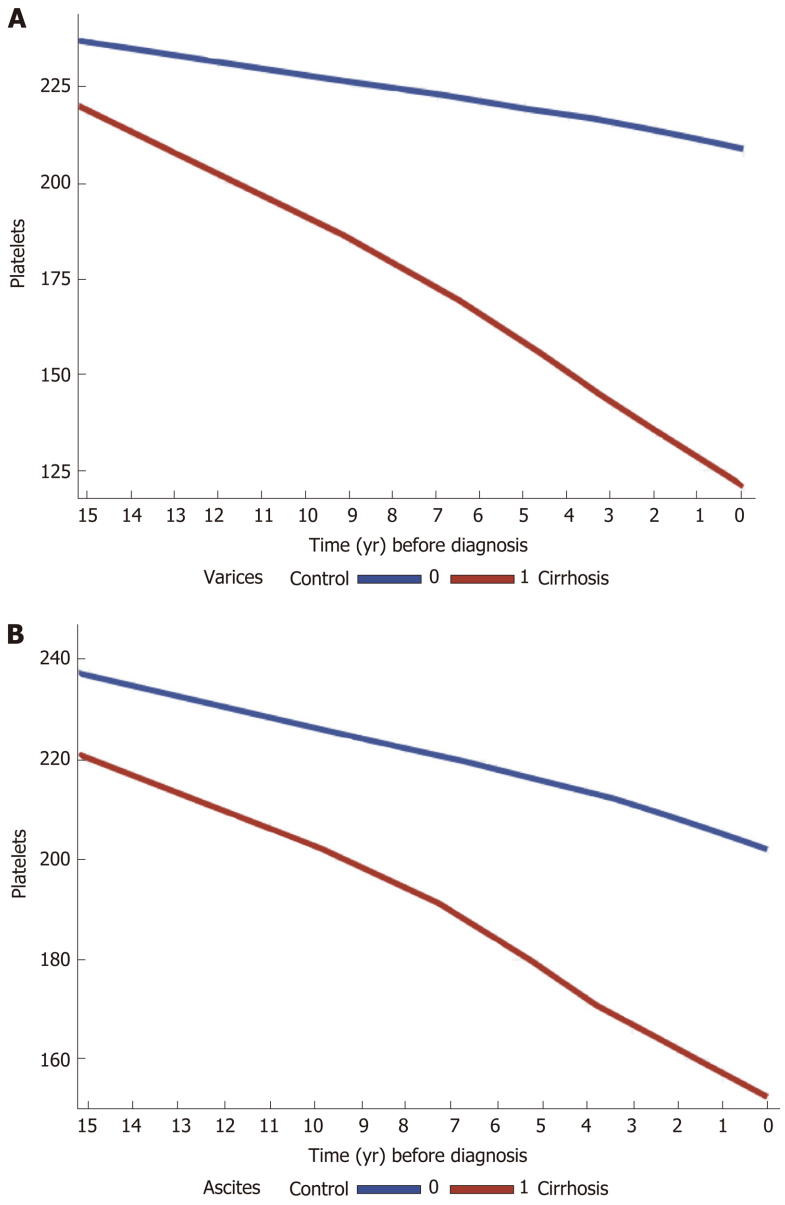

Figure 2 Trends in platelet counts across 15 years prior to cirrhosis diagnosis among cases and controls, stratified by cirrhosis complications A: Varices (*n* = 551) and B: Ascites (*n* = 450), both done with locally weighted scatterplot smoothing for trend. In patients with complications of portal hypertension (varices and ascites), platelet counts decline is steeper compared to those with no such complications.

and the number of measurements, we found that for every 50 units decrease in the platelets, the odds of cirrhosis increase by 1.3-fold (95%CI: 1.25-1.35).

DISCUSSION

Based on a large, well-characterized cohort, the results of this nested case-control study indicate that liver cirrhosis is characterized by a longitudinal decrement in platelet counts, within the normal limits, that may start 15 years prior to diagnosis. This trend is consistent regardless of sex, etiology of liver disease and observed after the age of 40.

In the cirrhosis group, more patients had diabetes mellitus (DM), CKD, a tendency for HTN and were smokers compared to controls. The relationship between these metabolic factors and CLD or cirrhosis is well established. However, their effect on PTC is unclear. We did not find a clear relationship between the presence of DM or HTN and lower PTC in the literature. The exact pattern of PTC in patients with CKD is controversial but several studies revealed a decrease in PTC and platelets dysfunction in renal failure[19-21]. The trend of PTC decrease was consistent in both sexes although

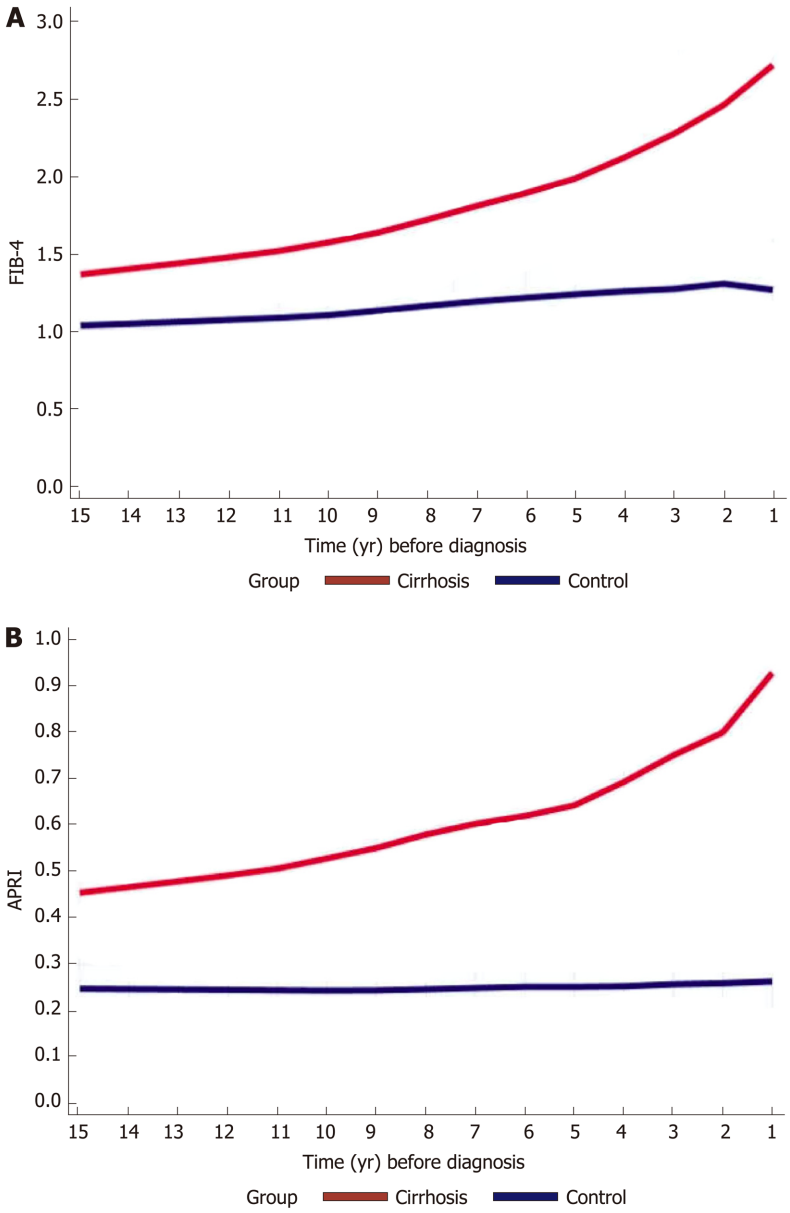

Figure 3 Trends in fibrosis-4 and aspartate aminotransferase-to-platelet ratio index scores across 15 years prior to cirrhosis diagnosis among cases and controls. Trends in A: Fibrosis-4 and B: Aspartate aminotransferase-to-platelet ratio index scores, both done with locally weighted scatterplot smoothing trend. There is a gradual increase in both scores in the cirrhosis group compared to controls, ranging from 1.3 to 3 and 0.48 to 0.93 respectively.

males had generally lower PTC values compared to females among both cases and controls. The literature on thrombocytopenia in liver disease does not show clear gender predominance so we can assume that this difference, in both groups, is physiological[22-25].

We compared other laboratory parameters that are related to CLD and portal HTN and examined the trends in the years prior to the diagnosis of cirrhosis. ALT, AST and GGT where above the upper normal limit (UNL) in the years preceding the diagnosis of cirrhosis, markedly so in the last two years before diagnosis, compared to controls in which liver enzymes were in the normal range. In both groups, there was a slight gradual increase in bilirubin and a slight decrease in albumin. These laboratory changes are probably physiological; several studies show small but incremental increases in bilirubin and a fall in serum albumin concentration that occurs with increasing age[26-29]. There was no change in the hemoglobin levels, while WBC decreased in cirrhotics compared to controls.

Due to the nature of the study we cannot ascertain why specific blood tests were taken and if liver disease was suspected by the treating physician which triggered more laboratory testing. Compared to the liver enzymes that were above to UNL and should have theoretically alerted the treating physician to the presence of liver disease,

at any given time or visit, the PTC during this period decreased within the normal range and were thus easy to miss.

The most common etiologies for cirrhosis in our cohort were viral hepatitis, ALD and NAFLD. The trend of decrease in PTC in our study was consistent regardless the etiology of liver cirrhosis. It was previously suggested, that different etiologies may be associated with different hepatic damage mechanisms relating to platelets which play a role in the induction of hepatic fibrosis[30,31]. A previous study in NAFLD, showed that PTC were decreased in cirrhotics over a 5 year follow up compared to healthy controls[14]. Additionally, a negative correlation between the PTC and the severity of liver fibrosis in NAFLD patients has been demonstrated; a linear decrease of the PTC was correlated with increasing histological fibrosis stage. A similar trend occurs in chronic HBV and HCV infection; previous studies showed that liver fibrosis in HCV patients, assessed by biomarkers and FibroScan®, was negatively correlated with PTC. Moreover, patients with advanced liver fibrosis had significantly lower PTC[32-36].

The platelets decrease trend in our study was most pronounced in patients with complications of liver cirrhosis and portal HTN with the steepest decline in patients who had varices. Studies show that together with liver and spleen stiffness measurements, PTC correlates with significant portal HTN and particularly in the presence of varices. The risk of having varices increases with decreasing PTC and have been used to assess the presence of varices non-invasively[37-40].

Our results suggest for the first time that the platelets trends can be used for the prediction of liver cirrhosis regardless the underlying etiology. In multivariate regression analysis, we found that for every 50 units decrease in the PTC, the odds of cirrhosis were 1.3 times higher. When looking at the normal range of PTC, a gradual decrease in PTC, still within the normal range, from 380 to 180 over time would signify 5.2- fold increase in the risk of being diagnosed with cirrhosis compared to individuals with no change in the PTC. Indeed, the progression to cirrhosis usually takes years to develop and may be missed due to lack of clinical symptoms or laboratory aberrations before significant portal HTN appears.

Diagnosis and staging of liver fibrosis are vital part of the clinical management of CLD of any etiology as it is associated with poor outcomes. Although liver biopsy is recommended as the gold standard for the diagnosis and staging of fibrosis, due to its invasive nature and other disadvantages, indirect assessments of liver fibrosis have been developed and are widely used. These include blood-based biomarkers (APRI, FIB-4, enhanced liver fibrosis, Fibro Test) and image-based techniques (US, transient elastography, shear wave elastography, Magnetic resonance elastography) as well as innovative methods that uses combined modalities including advanced magnetic resonance imaging sequences like diffusion-weighted magnetic resonance imaging and genetic testing[41,42].

Fibrosis risk scores have been developed based on readily available clinical and laboratory parameters that are simple to use at point of care, and can be implemented into computerized medical systems. However, current risk scores have several limitations; they incorporate PTC in the formulation, however, they do not consider progressive, longitudinal changes in PTC and use a single platelets value each time they are used[15,16,43-45].

Among others, FIB-4 have been most extensively studied and validated in diverse populations for the prediction of advanced fibrosis. Two cut-off values were defined; FIB-4 score ≤ 1.3 can be regarded as having a low risk for advanced fibrosis while score > 3.25 represents advanced fibrosis or cirrhosis. It was published previously, that intermediate FIB-4 values of 1.45–3.25 have negative predictive value of 89% for excluding advanced fibrosis and patients in this range would require a liver biopsy to assess the fibrosis stage. Thirty to forty percent of patients have an indeterminate score, and in these cases, additional testing is needed[35,46].

In our study, we show that along with the increase in AST (above the ULN) and age, a longitudinal PTC decrease before the diagnosis of cirrhosis, still within the normal range was associated in high FIB-4 and APRI scores, which were mostly in the range of 1.4-3.25, reaching values compatible with advanced fibrosis. Together with the intermediated values of FIB-4, the longitudinal PTC decrease, even within the normal levels, may reflect progressing fibrosis and can predict cirrhosis development. These combined changes may be picked up by computers and alert the treating physician of an ongoing liver disease before advanced fibrosis takes place, enabling therapeutic and preventing measures.

We acknowledge several limitations of this study. The main limitation is the retrospective nature of the study, with its built-in weaknesses of data collection and selection bias. Due to the nature of the study, we could not know why specific data was ordered/collected; especially which circumstances have led to the diagnosis of

cirrhosis. Additionally, clinical events may have been missed or only partly followed up, so that the diagnosis of cirrhosis could have been missed or not recorded. Additionally, we could not look at radiology or endoscopic results of each patient in order to identify signs of CLD, cirrhosis and portal HTN. All diagnoses were made exclusively according to ICD-9 codes. However, although we might have missed a large number of undiagnosed cirrhotic patients, our sample is large and representative enough to offer sound observations.

The number of platelets measurements and distribution in the preceding years before cirrhosis/sampling date was not equal. Cirrhotic patients had more PTC generally with the highest platelets measurements close to the diagnosis date. We hypothesize that there was a recognizable change in the medical condition of the patient which lead to more frequent tests. Due to the nature of this study, this information is not available. However, adjustment for the number and timing of testing did not attenuate the association.

Other limitations should also be noted. A relatively large proportion of patients in the cirrhosis group had missing data regarding the etiology of cirrhosis. However, this should not have an effect on the general observation.

This study also holds important strengths. We present longitudinal changes in PTC, compared to previous studies in which PTC were presented as a single measurement in a certain point of time. By using continuous and repeated measurements for the same individual, this method represented dynamic changes in laboratory data that indicated a trend before the diagnosis of cirrhosis. This method could potentially be used for longitudinal assessment of fibrosis regression following therapeutic interventions such as antiviral therapy or life style changes for NAFLD.

The recent interest in Big Data Mining, which is aimed at identifying patterns that are often unrecognizable during routine clinical management, enabled us to use the MHS database which offers high-quality data from electronic medical records, automatic data capture, and a central laboratory. The large number of members in this insurance group enabled the inclusion of a large study population both overall and in matched groups during a long period of time. Patients with various diseases (hematological/viral *etc*) and medications that could affect the PCT were excluded from the study so that the change in PTC could be attributed with high probability to the ongoing liver disease. Furthermore, our cohort represents a cohort of cirrhotic patients in a community and likely avoids selection bias seen in cohorts from tertiary referral centers.

CONCLUSION

Years before the diagnosis of liver cirrhosis is made there is a progressive decline in platelet counts, within the normal range, matched to a gradual increase in fibrosis scores. These changes may be identified by machine learning algorithms and alert the treating physicians of an early liver disease and may enable early therapeutic and preventive interventions before serious complications occur.

ARTICLE HIGHLIGHTS

Research background

Liver cirrhosis is usually asymptomatic early in its course. Many cirrhotic patients are diagnosed late when severe complications occur. A major challenge is to diagnose advanced fibrosis as early as possible, using simple and non-invasive diagnostics tools. Thrombocytopenia (platelet count < 150000/μL) on the background of chronic liver disease of any etiology represents advanced fibrosis and portal hypertension. As such, platelets have been incorporated in most non-invasive scores that predict liver fibrosis as a strong risk factor.

Research motivation

As opposed to thrombocytopenia which is associated with advanced fibrosis, little is known about the association between longitudinal changes in platelet counts (PTC), when still within the normal range, and the risk of cirrhosis.

Research objectives

To explore whether big data analysis of PTC trajectories over time, can predict advanced liver fibrosis and cirrhosis complications across the different etiologies of liver diseases.

Research methods

A nested case-control study with diagnosed cirrhosis patients and matched controls, utilizing the Maccabi Health Services database was performed. The trends of PTC, liver enzymes, bilirubin, international normalized ratio, albumin and fibrosis scores [fibrosis-4 (FIB-4) and aspartate transaminase-to-platelet ratio index] throughout the preceding 20 years prior to cirrhosis diagnosis were calculated and compared to healthy controls. The association between PTC, cirrhosis complications and fibrosis scores prior to cirrhosis diagnosis was investigated.

Research results

Cirrhosis cases (n = 5258) were compared to controls (n = 15744) matched for age and sex at a ratio of 1:3. The leading cirrhosis etiologies were viral, alcoholic and fatty liver disease. The mean PTC decreased from 240000/μL to 190000/μL up to 15 years prior to cirrhosis diagnosis compared to controls who's PTC remained stable around the values of 240000/μL. This trend was consistent regardless of sex, cirrhosis etiology and was more pronounced in patients who developed varices and ascites. Compared to controls whose values remained in the normal range, in the cirrhosis FIB-4 increased gradually from 1.3 to 3 prior to cirrhosis diagnosis. Additionally, in multivariable regression analysis, a decrease of 50 units in PTC was associated with 1.3 times odds of cirrhosis (95%CI: 1.25-1.35).

Research conclusions

This study indicates that a progressive decline in platelet counts, within the normal range, is associated with a gradual increase in fibrosis scores, starting up to 15 years before the diagnosis of cirrhosis.

Research perspectives

Progressive PTC decline in the preceding years before the diagnosis of liver cirrhosis, when still within the normal limits, may be identified by machine learning algorithms and alert the treating physicians of an early liver disease and may enable early therapeutic and preventive interventions before serious complications occur.

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ORIGINAL ARTICLE

Retrospective Study

Endoscopic ultrasound-measured muscular thickness of the lower esophageal sphincter and long-term prognosis after peroral endoscopic myotomy for achalasia

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Institutional review board statement: This study was

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Abstract

BACKGROUND

People with achalasia typically have a thick lower esophageal muscularis propria (LEMP), and peroral endoscopic myotomy (POEM) has been effective in treating most patients. LEMP thickness may be associated with the outcomes and prognosis after POEM. However, more evidence is needed regarding the relationship between LEMP thickness and patient prognosis after POEM.

AIM

To assess the association between LEMP thickness, measured using endoscopic ultrasound (EUS), and long-term prognosis, especially relapse, after POEM for achalasia.

METHODS

All medical records, including EUS data, of patients who underwent POEM to treat achalasia at Shengjing Hospital of China Medical University from January 2012 to September 2018 were retrospectively reviewed. LEMP thickness was measured by EUS, and a thickness of ≥ 3 mm was defined as thickened. The severity of patient symptoms was evaluated using the Eckardt score. Relapse was defined as a 3-point rise in the Eckardt score after a period of clinical remission. The relationship between patient characteristics, muscle thickness, and recurrence was analyzed.

RESULTS

reviewed and approved by the Institutional Review Board and the Ethics Committee of China Medical University.

Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

Conflict-of-interest statement: We have no financial relationships to disclose.

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Eighty-two patients (32 males and 50 females, aged 17-78 years) and 85 POEM procedures were included. In total, 76.8% (63/82 patients) of patients had a thickened muscularis propria. Older age and longer disease course were associated with muscularis propria thickening ($P < 0.05$). The mean postoperative follow-up time was 35.4 ± 17.2 mo (range, 8-87.5 mo) in 60 patients. Five patients with Eckardt scores > 3 refused further management after their symptoms were relieved. The relapse rate was 12.73% (7/55 cases). Five patients, four of whom had muscularis propria thickening, had disease recurrence within 12 mo after the procedure. Achalasia relapsed in one patient who had a thickened muscularis propria after 24 mo and in another patient who did not have a thickened muscularis propria after 30 mo. Patients with recurrence were typically younger and had a shorter disease course ($P < 0.05$). The relapse rate in patients with a non-thickened muscularis propria tended to be higher (18.2%, 2/11 patients) than that in patients with a thickened muscularis propria (11.4%, 5/44 patients), although no significant difference was found. Age (hazard ratio = 0.92; 95% confidence interval: 0.865-0.979; $P < 0.05$) and being male (hazard ratio = 7.173; 95% confidence interval: 1.277-40.286; $P < 0.05$) were identified as risk factors for symptomatic recurrence by multivariable analysis using the Cox model.

CONCLUSION
Patients with a thickened muscularis are typically older and have a longer disease course. Younger age and the male sex are associated with increased recurrence. Patients with a thin muscularis propria may be prone to relapse, although further validation is needed.

Key Words: Peroral endoscopic myotomy; Achalasia; Endoscopic ultrasound

Core Tip: This study investigated the clinical significance of lower esophageal muscularis propria (LEMP) thickness in achalasia patients. We retrospectively enrolled 82 patients who underwent peroral endoscopic myotomy (POEM) at our center. The results showed that endoscopic ultrasound-measured LEMP thickness may help diagnose achalasia and predict long-term prognosis after POEM. Patients with a thickened muscularis were typically older and had a longer disease course. Younger age and male gender were risk factors for recurrence. Achalasia was more likely to relapse after POEM in patients with a thin LEMP, although further validation is needed.

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INTRODUCTION

Achalasia is currently the most common primary esophageal motility disease[1]. While its etiology remains uncertain, achalasia is characterized by the destruction of inhibitory ganglion cells in the myenteric plexus, causing severe myopathy in smooth muscles and leading to aperistalsis and impaired relaxation of the lower esophageal sphincter (LES)[1,2]. In addition, thickened lower esophageal muscularis propria (LEMP) has been observed in individuals with achalasia *in vivo* and during autopsies[3-7]. LEMP hypertrophy may be a response to esophageal functional obstruction or a primary lesion[2]. Hence, LEMP thickness is thought to be associated with the disease state.

Existing treatments for achalasia, including pharmacotherapy, clostridium botulinum injection, endoscopic balloon catheter dilation, and Heller myotomy, target the compulsory release of the stenosis segment of the LES. Peroral endoscopic

myotomy (POEM) has emerged as an increasingly utilized treatment approach, especially in patients with multiple comorbidities who cannot undergo laparoscopic or open surgical interventions[8,9]. POEM is considered safe for patients as young as 3 years old, with no upper age limit, according to several efficacy studies with a maximum follow-up period of 3 years[10-13].

Despite its safety, patients who undergo POEM are at risk for disease recurrence[14-16], although there is no recognized predictor. However, treatment history, type of mucosal damage, and reflux symptoms were included in a newly developed risk prediction scoring system[17]. Considering that the POEM procedure is performed directly on the muscularis propria and LEMP thickness is associated with the postprocedural balloon catheter dilation outcome[7], LEMP thickness is thought to be associated with POEM outcome. However, no correlation between muscle thickness and prognosis at 1 year after POEM has been reported[18,19], and few studies with longer follow-up periods include assessment of the relationship between LEMP thickness and POEM outcomes.

Therefore, this study aimed to examine the relationship between LEMP thickness, measured by endoscopic ultrasound (EUS), and patient characteristics including long-term prognosis after POEM for achalasia at our health center in the past 7 years.

MATERIALS AND METHODS

Patients

All medical records of patients who underwent POEM to treat achalasia at Shengjing Hospital of China Medical University from January 2012 to September 2018 were retrospectively reviewed. The inclusion criteria were diagnosis of achalasia and record of LEMP thickness measured by EUS examination before POEM. The exclusion criteria were as follows: (1) History of gastrointestinal open surgery; (2) Gastrointestinal malignancy; (3) Coagulopathy or other systemic disorders that precluded safe use of general anesthesia; and (4) Unwillingness to provide informed consent.

From the patient medical charts, demographic information, disease duration defined by time of symptom onset to POEM procedure, symptoms, Eckardt scores[20], degree of esophageal dilation based on the upper gastrointestinal tract X-ray, endoscopy findings, thickness of LEMP according to EUS findings, and treatment outcomes were collected for analysis.

Ethics

This study was approved by the Institutional Review Board and the Ethics Committee of China Medical University. All patients voluntarily chose their therapeutic course and provided written informed consent for the POEM procedure. Written informed consent was obtained from the parents or guardians of patients younger than 18 years of age.

Symptom evaluation

Clinical symptoms of all patients were evaluated according to the Eckardt score before and after the procedure. The Eckardt score is the sum of the symptom scores for dysphagia, regurgitation, retrosternal pain (with a score of 0 indicating the absence of symptoms, 1 indicating occasional symptoms, 2 indicating daily symptoms, and 3 indicating symptoms at each meal), and weight loss (with 0 indicating no weight loss, 1 indicating a loss of < 5 kg, 2 indicating a loss of 5-10 kg, and 3 indicating a loss of > 10 kg)[20]. Therefore, scores could range from 0 to 12, with 0 representing no symptoms.

EUS evaluation

All EUS evaluations were performed by experienced technicians using a 360° Radial-Array Ultrasound Gastroscope (EG-3870URK; PENTAX Medical, Tokyo, Japan) and an ultrasound scanner (EUB 6500; Hitachi, Tokyo, Japan). The thickness of the muscularis propria at the esophagogastric junction (EGJ) was assessed before POEM, and a thickness of ≥ 3 mm was considered thickened. The cutoff value for the muscular thickness was determined according to the ordered sample cluster method based on age, disease duration, preoperative Eckardt score, and postprocedural outcome. The completeness of the myotomy was verified by EUS after POEM.

Endoscopic procedure of POEM

POEM procedures were performed by three experienced therapeutic endoscopists.

Patients fasted for 24-48 h and were forbidden from drinking water for 4-6 h before the procedure. If gastroscopy or EUS revealed any liquid or food residue in the esophagus, a decompression tube was indwelled for at least 24 h before POEM, and nutrients were introduced intravenously. During the procedure, patients were placed in the left recumbent position under general anesthesia with tracheal intubation. The POEM procedure was performed as follows: (1) A submucosal injection (Boston Scientific, United States) of a mixture of saline and methylene blue was administered into the esophageal wall at 12-15 cm above the EGJ; (2) A submucosal tunnel passing over the EGJ was created using a hook knife (KD-620LR; Olympus Corp., Tokyo, Japan) or a triangle tipped knife (KD-640L; Olympus) extending 3 cm into the proximal stomach; (3) Inner circular myotomy began 2-3 cm below the tunnel entry and ended at the cardia; and (4) After careful hemostasis using hemostatic clips (FD-410LR; Olympus), several metal clips [ROOC-D-26-195; Micro-Tech (Nanjing) Co., Ltd., Jiangsu, China] were applied to close the mucosal entry. CO_2 was used as the endoscope air supply. Prophylactic antibiotics and proton pump inhibitors were administered intravenously for at least 2 d after the procedure.

Assessment of therapeutic response and follow-up

Follow-up for all patients was conducted by telephone calls or clinic visits, and clinical symptoms were assessed using the Eckardt score. The procedure was considered effective, and the patient was in clinical remission if the postoperative Eckardt score was ≤ 3. Relapse was defined as a rise in the Eckardt score to > 3 after a period of clinical remission[15]. For patients who underwent the procedure twice, the first set of data was used to analyze the disease recurrence rate.

Statistical analysis

Statistical analyses were performed using SPSS version 25.0 software (IBM, Armonk, NY, United States). Normally distributed continuous variables are expressed as the mean ± SD and were compared using a *t*-test. Categorical data were compared using the chi-square test and are expressed as numbers (percentages). The association between age and disease duration was analyzed by univariate logistic regression analysis. Recurrence-free survival was investigated using the Kaplan–Meier estimate of time-to-event and compared using a log-rank test. After confirming that the possible risk factors satisfied the proportional hazard hypothesis, the Cox proportional hazards model was used to evaluate risk factors for clinical recurrence. $P < 0.05$ was considered statistically significant. The ordered sample cluster analysis was performed using DPS version 7.05 software (Zhejiang University, Hangzhou, China)[21].

RESULTS

Muscle features of the lower esophageal sphincter in patients with achalasia

Eighty-two patients (32 males and 50 females aged 17-78 year with a mean age of 46.5 ± 14.9 year) and a total of 85 procedures were included. The average disease duration was 102.4 ± 127.2 mo (range, 1-516 mo). All patients denied having a previous diagnosis of esophageal disease. Six patients had a history of an endoscopic procedure. Of all patients, 63 (76.8%) had a thickened LEMP.

Data on the correlation between patient background and LEMP thickness are shown in Table 1. Significant differences in age (48.4 ± 14.4 years *vs* 40.4 ± 15.2 years, $P < 0.05$) and disease duration (126.63 ± 138.49 mo *vs* 37.79 ± 35.52 mo, $P < 0.05$) were observed between the thickened and non-thickened LEMP groups, and both variables were associated with thickened LEMP. Specifically, older patients with longer disease duration were more likely to have a thickened LEMP. In addition, there was a positive correlation between patient age and duration of the disease (Figure 1).

The degree of esophageal dilation in 53 patients was evaluated, while the rest of the patients underwent barium esophagraphy at other medical centers and were not included. Of these, 21 (39.6%), 24 (45.3%), and 8 (15.1%) patients exhibited degrees I, II, and III dilations, respectively.

Therapeutic effect of POEM and long-term prognosis

The overall POEM success rate was 94.12% (80/85 procedures). There was only one reported case of post-procedure infection, which was resolved using conservative treatment. The EUS data confirmed complete myotomy after POEM in 62 cases. Sixty patients had follow-ups between 8 and 87.5 mo (mean: 35.4 ± 17.2 mo)

Table 1 Comparison of characteristics between patients with non-thickened and thickened lower esophageal muscularis propria

	Thickness of LEMP		*P* value
	< 3 mm (*n* = 19)	≥ 3 mm (*n* = 63)	
Age (yr)	40.4 ± 15.2	48.4 ± 14.4	0.04
Sex (male/female)	6:13	26:37	0.448
Disease duration (mo)	37.79 ± 35.52	126.63 ± 138.49	< 0.001
History of endoscopic surgery (+/-)	1:18	5:58	0.619
Dilation grade (I/II/III)	6:4:1 (11)	15:20:7 (42)	0.667
Preoperative Eckardt score	6.53 ± 2.04	6.68 ± 1.90	0.758

LEMP: Lower esophageal muscularis propria.

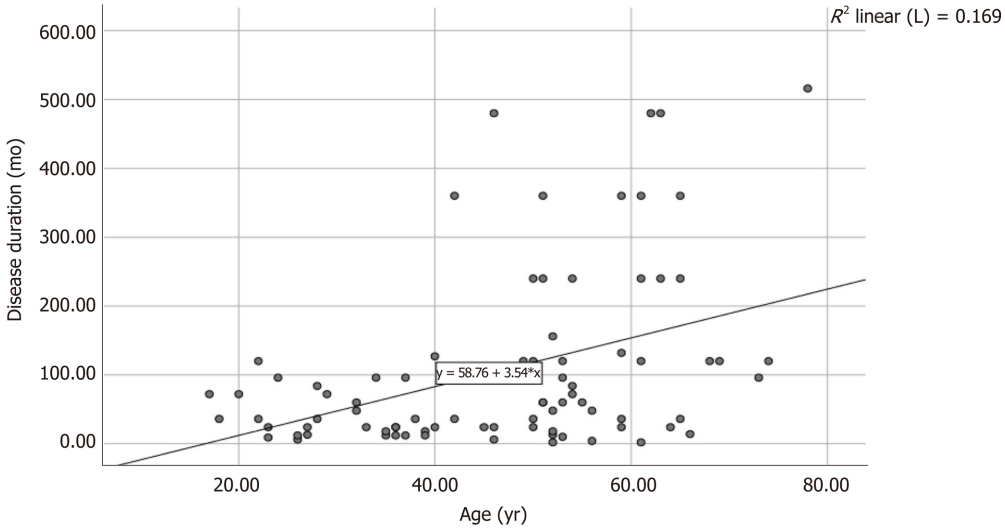

Figure 1 Correlation between age and disease duration in individuals with achalasia ($P < 0.05$, $R^2 = 0.169$).

after the procedure. Five patients with Eckardt scores > 3 refused further follow-up after their symptoms were relieved. There were no significant differences in age, sex, disease duration, preoperative Eckardt score, or LEMP thickness between the effective (n = 55) and ineffective (n = 5) groups. The relapse rate was 12.73% (7/55 cases). Five patients, four of whom had a thickened LEMP, had symptom recurrence within 12 mo after the procedure. One patient who had a thickened LEMP relapsed after 24 mo while another, who did not have a thickened LEMP, relapsed after 30 mo. Overall, the effectiveness (absence of relapse) of POEM was 87.3% up to 87 mo postoperatively (Figure 2).

Patients with symptomatic recurrence were younger and had shorter disease duration. The relapse rate in patients without a thickened LEMP tended to be higher than patients with a thickened LEMP (2/11 patients *vs* 5/44 patients). However, no significant difference was found. Data on patient characteristics according to POEM outcomes are shown in Table 2 and Figure 3. Younger age (hazard ratio = 0.92; 95% confidence interval: 0.865-0.979; $P < 0.05$) and male gender (hazard ratio = 7.173; 95% confidence interval: 1.277-40.286; $P < 0.05$) were found to be associated with recurrence according to Cox regression analysis (Table 3).

DISCUSSION

Although several previous studies have demonstrated that LEMP thickness is associated with POEM procedure duration and outcomes, the present study adds to the literature by specifically examining the relationship between LEMP thickness and

Table 2 Comparison of characteristics in patients with different outcomes after peroral endoscopic myotomy

	Not effective (*n* = 5)	Clinical success/effective (*n* = 55)		*P* value
		No relapse (*n* = 48)	Relapse (*n* = 7)	
Age (yr)	33.8 ± 11.97	48.02 ± 14.73	34.00 ± 13.05	0.021[a]
Sex (male/female)	3:2	16:32	4:3	0.280
Disease duration (mo)	82.8 ± 60.36	117.29 ± 131.41	35.57 ± 30.42	0.001[a]
Preoperative Eckardt score	6.60 ± 2.40	6.65 ± 1.74	7.57 ± 2.23	0.209[a]
Verification of myotomy by EUS (Yes/No)[1]	3:2	39:11	6:1	0.539
LEMP (thickened/ non-thickened)	4:1	39:9	5:2	0.832

[1]Based on procedure number.
[a]*P* < 0.05, between relapse and no relapse. LEMP: Lower esophageal muscularis propria; POEM: Peroral endoscopic myotomy; EUS: Endoscopic ultrasound.

Table 3 Risk factor analysis for recurrence using the Cox proportional hazards model

Variable	Univariate		Multivariate	
	Hazard ratio (95%CI)	*P* value	Hazard ratio (95%CI)	*P* value
Age	0.943 (0.893-0.995)	0.033	0.920 (0.865-0.979)	0.008
Male sex	2.829 (0.629-12.712)	0.175	7.173 (1.277-40.286)	0.025
Disease duration	0.984 (0.963-1.006)	0.144	NA	0.402
Preoperative Eckardt score	1.296 (0.855-1.965)	0.222	-	-
LEMP ≥ 3 mm	0.606 (0.117-3.128)	0.550	-	-

CI: Confidence interval; LEMP: Lower esophageal muscularis propria; NA: Not assessed; -: Not included for analysis; CI: Confidence interval.

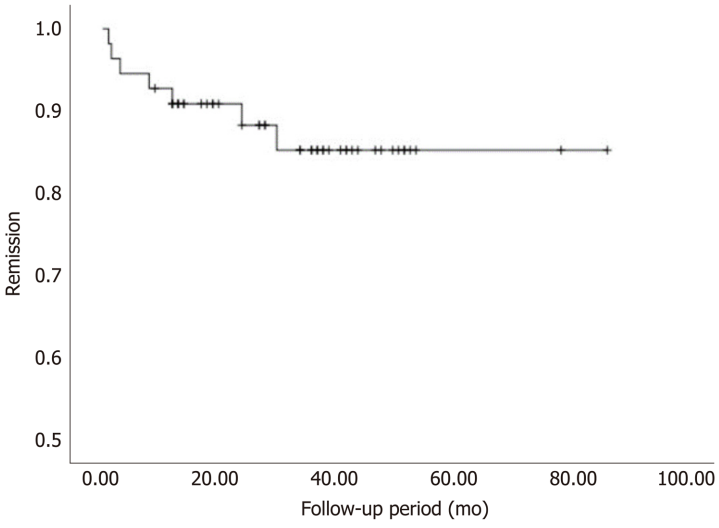

Figure 2 Kaplan–Meier analysis of recurrence after peroral endoscopic myotomy.

long-term outcomes, including disease relapse after POEM. The results from the present retrospective chart review and statistical analysis suggest that non-thickened LEMP may be associated with increased disease recurrence. In addition, there is a correlation between older age, longer disease duration, and thickened LEMP.

In previous reports, no significant correlation has been found between the severity of achalasia evaluated by Eckardt score and LEMP thickness[19,22]. However, age, pneumatic dilation history, and male sex have been reported as predictors of a

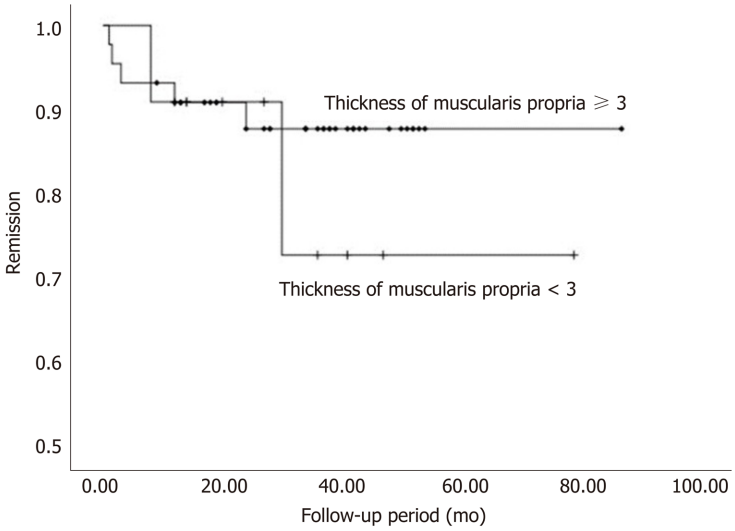

Figure 3 Kaplan–Meier estimate of time to recurrence according to muscularis propria thickness.

thickened LEMP in several studies[6,19,22,23]. Similarly, the present study showed a positive association between age and muscle thickness. In addition, the present analysis found an association between disease duration and LEMP thickness, as patients with a thickened LEMP had a significantly longer disease course. The results may be attributed to differences in patient characteristics among the studies as we also found a positive association between age and disease duration. Thus, the thickened LEMP could be due to prolonged exposure to reactive hyperplasia since longer disease duration means longer stimulation time. The reversibility of the LES hypertrophy after POEM provides supporting evidence for this theory[18].

The thickness of the LEMP may be associated with procedural outcomes and postoperative prognosis. We were not able to statistically evaluate the relationship between POEM-related complications and muscle thickness due to the small sample population. Previous studies have shown, without statistical significance, that there tended to be a positive association between muscle thickness, procedure duration, and complication rate[24]. As for postoperative prognosis, symptomatic recurrence has been reported to occur between 2-5 years after POEM[14]. A medium-term recurrence rate of 18% was reported in a 2-year follow-up study. Although no explanations for recurrence were given, there was an association between younger age and increased rate of relapse[16]. Watanabe *et al*[18] reported no significant difference in clinical outcomes between patients with thick and those with thin LEMP in a 1-year follow-up study, observing no cases of recurrence. Li *et al*[15] reported a cumulative recurrence rate of 13.7% at 5 years post-POEM that correlated with disease duration and interventional treatment history. In the same study, age and symptom severity showed no correlation with achalasia relapse.

Since the analysis of the present study confirmed a relationship between patient characteristics and LEMP thickness, the effect on recurrence was also analyzed. Although there was no statistical significance, there was a slightly higher relapse rate in patients with non-thickened LEMP. The lack of statistical significance may be due to the treatment of the muscle thickness measurements as discontinuous data. Patients with a younger age and shorter disease duration had an increased rate of relapse. This may be explained by the better healing ability in younger individuals and the fact that patients with shorter disease duration did not have adequate reactive hyperplasia of the LES after esophageal functional obstruction. Our results may suggest that POEM in patients with a certain LEMP thickness caused by reactive hyperplasia has a lower disease recurrence rate.

Cox analysis showed that disease duration was not associated with recurrence, whereas age remained an effective predictor. This may be explained by the co-linear relationship between age and disease duration. Male sex was also a risk factor for recurrence, although the large confidence interval may indicate that this was a result of a sampling error. Besides, the extent of myotomy was not associated with POEM prognosis[25]. Therefore, our result is still reliable, although we are not able to discuss the effect of the degree of myotomy on prognosis after POEM as all patients enrolled underwent myotomy of the same degree in this study. Our result, which shows that

younger age is associated with increased recurrence, concurs with the findings of Werner *et al*[16] but contradicts those of Li *et al*[15,16]. Therefore, the association between age, disease duration, and relapse after POEM remains unclear, and further large-scale validation studies are needed.

The Chicago classification, based on high-resolution manometry, is a predictor of achalasia treatment outcomes. Subtype II has the best prognosis, whereas the prognosis of subtype I is slightly poorer and subtype III can be difficult to treat[26]. Patients within the achalasia subtypes tend to have similar LEMP thicknesses[19,24]. The difference in prognostic efficacy between LEMP thickness and subtype is still unclear. However, some participants in our study were not available for manometry. To include a larger sample size in the present study, we did not evaluate the relationship between Chicago classification and POEM prognosis, nor did we compare the subtypes with LEMP thickness. We expect future studies to reveal the relationship between achalasia subtypes, LEMP thickness, and POEM prognosis.

LEMP thickness may help in diagnosing achalasia. A thickened LEMP in patients with achalasia was first observed in an autopsy[4] and with the development of EUS, measurement of the intrinsic muscle layer thickness *in vivo* is feasible[8,27-30]. Using a 7.5-MHz ultrasound endoscope, Devière *et al*[3] reported a thickened LEMP in individuals with achalasia compared with healthy controls. Conversely, Ponsot *et al*[31] argued that those results may have been attributable to puckering of the EGJ and found that the LEMP was not thicker in patients with achalasia than in healthy controls. In the 30 years since these studies, the number of studies that use different EUS techniques and include more participants have increased. Our results agree with many of these studies, where most of the individuals in the present study had a thickened LEMP[5-7]. This suggests that LEMP thickness may be of value in achalasia diagnosis. However, because the identification of the differences in LEMP structure between patients with achalasia and healthy individuals is not easy, the use of muscle thickness in diagnosing achalasia is still controversial.

Our study has several limitations. First, it was done retrospectively and without a healthy control group for comparison of LEMP thickness according to age and other patient characteristics. Second, there was no protocol for measuring the esophageal wall thickness and variations in measurements have been noted in previous studies. Third, evaluation of disease severity and recurrence using the Eckardt score can be highly subjective where physicians may tend to report more marked symptom improvement than patients[32]. Fourth, the cross-sectional area (CSA) of the muscularis propria may be a better indicator to estimate the degree of hypertrophy since a disassociation between muscle thickness and the CSA in some individuals with a dilated esophagus was observed. Patients with a dilated and distended esophagus may have a thin LEMP but a high CSA because of distension of the esophageal wall[5]. Fifth, although the normal value of LEMP thickness is unknown, the cutoff of 3 mm is higher than that in previous studies[3,23,24]. This suggests that our patient population may not be the best representative of Achalasia patients in general, a fact that might undermine the external validity of the results. However, considering the difference of age and disease duration in patients with different LEMP thicknesses, we believed that the 3 mm cutoff in this study is reasonable. In addition, the pathophysiology of achalasia is not fully understood and the role of muscular features in the progression and prognosis of the disease is still undetermined. Therefore, future studies should focus on clarifying the etiology of the disease to explain the mechanisms associated with its pathophysiology.

CONCLUSION

Most patients with achalasia have a thickened muscularis propria determined by EUS measurement. Therefore, it is likely that EUS can help diagnose achalasia. Patients with a thickened muscularis are typically older and had a longer disease course. Younger age and male gender are associated with recurrence in the present study. Patients with a thin muscularis propria may have a higher risk of symptomatic recurrence, but validation through further large-scale studies is needed.

ARTICLE HIGHLIGHTS

Research background

Peroral endoscopic myotomy (POEM) procedure is an effective treatment for achalasia as it cuts open the lower esophageal muscularis propria (LEMP) directly. LEMP thickness of achalasia patients may be associated with the outcomes and prognosis after POEM.

Research motivation

Patients who undergo POEM are at risk for disease recurrence. Several predictors have been reported while the relationship between LEMP thickness and long-term prognosis after POEM is unclear.

Research objectives

In this retrospective study, we analyzed the relationship between LEMP thickness, patient characteristics, and long-term prognosis after POEM in individuals who underwent POEM for achalasia at our health center in the past seven years.

Research methods

All medical records, including EUS data, of patients who underwent POEM to treat achalasia at Shengjing Hospital of China Medical University from January 2012 to September 2018 were retrospectively reviewed. The severity of patient symptoms was evaluated using the Eckardt score. Relapse was defined as a 3-point rise in the Eckardt score after a period of clinical remission. The relationship between patient characteristics, muscle thickness, and recurrence was analyzed.

Research results

Older age and longer disease course were associated with muscularis propria thickening ($P < 0.05$). Patients with recurrence were typically younger and had a shorter disease course ($P < 0.05$). The relapse rate in patients with a non-thickened muscularis propria tended to be higher (18.2%, 2/11 patients) than patients with a thickened muscularis propria (11.4%, 5/44 patients), although no significant difference was found. Age (hazard ratio = 0.92; 95% confidence interval: 0.865-0.979; $P < 0.05$) and male sex (hazard ratio = 7.173; 95% confidence interval: 1.277-40.286; $P < 0.05$) were identified as risk factors for symptomatic recurrence by multivariable analysis using the Cox model.

Research conclusions

Patients with a thickened muscularis are typically older and have a longer disease course than those without a thickened muscularis. Younger age and male sex are associated with increased recurrence. Patients with a thin muscularis propria may be prone to relapse, although further validation is needed.

Research perspectives

A large-scale prospective study should be conducted to gain more evidence for the relationship between achalasia subtypes, LEMP thickness, and POEM prognosis.

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ORIGINAL ARTICLE

Observational Study

Monitoring hepatitis C virus treatment rates in an Opioid Treatment Program: A longitudinal study

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Author contributions: Sanvisens A and Muga R designed the study and wrote the first draft of the manuscript; Sanvisens A managed the literature searches and statistical analysis; Rivas I, Faure E, Espinach N, and Hernandez-Rubio A recruited the study population and took care of patients; Sanvisens A, Rivas I, Majó X, Colom J and Muga R reviewed the literature and made contributions to the interpretation of data; and all the authors contributed to the discussion section and revised and approved the final manuscript.

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Abstract

BACKGROUND

Direct-acting antivirals (DAAs) are recommended for the treatment of hepatitis C virus (HCV) infection in patients treated with methadone or buprenorphine.

AIM

To assess HCV treatment rates in an Opioid Treatment Program (OTP).

METHODS

This longitudinal study included 501 patients (81.4% men, median age: 45 years; interquartile range: 39-50 years) enrolled in an OTP between October 2015 and September 2017. Patients were followed until September 2019. Data on socio-demographics, substance use, HCV infection, human immunodeficiency virus (HIV) infection and laboratory parameters were collected at entry. We analyzed medical records to evaluate HCV treatment. Kaplan-Meier methods and Cox regression models were used to analyze the DAA treatment uptake and to identify treatment predictors.

RESULTS

Prevalence of HCV and HIV infection was 70% and 34%, respectively. Among anti-HCV-positive (n = 336) patients, 47.2%, 41.3%, and 31.9% used alcohol,

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Institutional review board statement: The study was reviewed and approved by the Ethics Committee of the Hospital Universitari Germans Trias i Pujol (PI-15-100), Badalona, Spain.

Informed consent statement: All study participants, or their legal guardian, provided written consent prior to study enrollment.

Conflict-of-interest statement: The authors of this manuscript having no conflicts of interest to disclose.

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cannabis, and cocaine, respectively. HCV-RNA tests were positive in 233 (69.3%) patients. Twentyeight patients (8.3%) cleared the infection, and 59/308 (19.1%) had received interferon-based treatment regimens before 2015. Among 249 patients eligible, 111 (44.6%) received DAAs. Treatment rates significantly increased over time from 7.8/100 person-years (p-y) (95%CI: 5.0-12.3) in 2015 to 18.9/100 p-y (95%CI: 11.7-30.3) in 2019. In a multivariate analysis, patients with HIV co-infection were twice as likely to receive DAAs (HR = 1.94, 95%CI: 1.21-3.12) than patients with HCV mono-infection. Current drug use was an independent risk factor for not receiving treatment against infection (HR = 0.48, 95%CI: 0.29-0.80).

CONCLUSION
HCV treatment is evolving in patients with HCV-HIV co-infection. Ongoing drug use while in an OTP might negatively impact the readiness to treat infection.

Key Words: Direct-acting antiviral agents; Opioid Treatment Program; Opioid agonist therapy; Hepatitis C virus infection; Human immunodeficiency virus infection; Drug use

Core Tip: Longitudinal study carried out in the only Opioid Treatment Program authorized for the provision of methadone or buprenorphine in a large urban area of 360000 inhabitants. Results indicate that hepatitis C virus treatment rates are increasing since the introduction of direct antiviral agents and identifies gaps and challenges on the readiness to treat infection.

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INTRODUCTION

It is estimated that 10 million people with substance use disorder (SUD) have hepatitis C virus (HCV) infection[1]. In addition, it is believed that a proportion of HCV infections remain undiagnosed in individuals with SUD. According to the World Health Organization (WHO), 23% of new HCV infections occur in patients with SUD[2]. In the United States and western Europe, two out of every three new HCV infections are believed to be associated with substance use[2].

The introduction of direct-acting antiviral agents (DAAs) in 2013 caused substantial changes in the clinical outcomes of HCV infection. Pharmacotherapy for HCV infection is administered for shorter periods of time (*i.e.*, 8-12 wk) and sustained virological responses (SVR) are achieved in over 90% of patients, irrespective of the HCV genotype. Several studies have revealed that DAAs showed efficacy in difficult-to-treat populations, including individuals with SUD[3-8].

The WHO aims to eliminate HCV infection by 2030. The defining features of that goal are to achieve a 90% reduction in new cases, diagnose 90% of all individuals infected with HCV, treat 80% of those eligible, and reduce death by 65%. In this context, individuals with SUD have been recognized as a target population for improving the identification of HCV-related disease and for implementing HCV micro-elimination strategies[9-11]. The strategy is to promote a cascade of care, or a continuum of services that should be provided to cure HCV in persons living with hepatitis[2].

Current guidelines for HCV care and treatment are provided, among others, by the American Association for the Study of the Liver (AASLD), the European Association for the Study of the Liver (EASL), and the WHO[12-14]. All of these organizations recommend DAAs for treating HCV infection, including in individuals with SUD. Indeed, several studies have indicated that SUDs did not affect adherence to treatment or imply worse response rates[15-17].

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More than 120000 people have been treated with DAAs since the Strategic Plan for Tackling Hepatitis C was implemented by the Spanish National Health System in 2015[18]. At the same time, up to 60000 patients are regularly treated with opioid agonist therapy (*i.e.*, methadone) in Spain. Individuals treated with methadone might have a history of injected drug use, and consequently, they might have acquired blood-borne infections, like HCV, after they began injecting drugs[19]. A previous study on individuals that participated in Opioid Treatment Programs (OTPs) in Catalonia, Spain, showed that the prevalences of HCV and human immunodeficiency virus (HIV) infections were 74% and 54%, respectively[20].

We hypothesized that in the context of the changes made in the provision of HCV care, OTP sites might be experiencing increasing proportions of patients that are eligible for HCV treatment. Therefore, we studied OTP participants to analyze assessment of infection, treatment rates, and predictors of treatment with DAAs.

MATERIALS AND METHODS

This longitudinal study included ex-heroin users enrolled in an OTP between October 2015 and September 2017. The OTP operates in a municipal outpatient clinic specialized in the treatment of SUDs in Badalona (240000 inhabitants) and Santa Coloma de Gramenet (120000 inhabitants), Spain. The selection process of the study population was conducted in the only addiction clinic for the provision of methadone in both cities during the study period.

In the OTP, methadone is dispensed on site, *via* a mobile unit (Intercity Methadone Bus), and in five community pharmacies. In addition, the outpatient clinic conduct harm reduction programs, which include needle exchanges, condom distribution, and psychosocial interventions[21].

For OTP inclusion, patients had to be over age 18 years and they had to have an opioid dependence diagnosis, based on the Diagnostic and Statistical Manual of Mental Disorders, 4th edition criteria[22]. Additional details have been described previously[20,21]. The municipal clinic was affiliated with primary care centers and nearby hospitals, where patients were referred for confirmatory tests (*e.g.*, HCV-RNA), radiology (*e.g.*, ultrasound), and consultations with specialists (*e.g.*, hepatologists). Physicians at the OTP clinic did not evaluate liver disease or treat HCV infection; those patients were referred to the hospital, where hepatologists and/or internist treated HCV infection. The Spanish health system provided universal access to DAAs, but these drugs were only dispensed in hospital pharmacies.

Ethics

Patients were informed of the objective of the study, and all patients provided written consent. The study was approved by the Ethics Committee of the Hospital Universitari Germans Trias i Pujol (PI-15-100). This study was compliant with ethical standards for medical research and good clinical practice principles, and it was performed in accordance with the World Medical Association's Declaration of Helsinki.

Variables

We collected data on socio-demographic variables (education level, employment, and prior imprisonment), opioid use (age at first drug use, main route of administration), biochemistry and hematological parameters, including liver function tests (aspartate aminotransferase, alanine aminotransferase, gamma glutamyl transferase, and total bilirubin). We also ascertained the presence of HIV and HCV infections and HCV-RNA, the genotype, and any antecedent of HCV treatment with interferon-based regimens.

Follow-up

Patients that tested anti-HCV positive and had not previously received IFN/RBV treatment regimens were followed-up until September 30, 2019. Specifically, we reviewed clinical charts to ascertain data on HCV-RNA, the genotype, and DAA treatments, including the date of initiation, type, duration, and clinical outcome (*i.e.*, SVR). In addition, we checked the national death registry for all patients.

Statistical analysis

We performed a descriptive analysis of the data. Continuous variables are presented as the median and interquartile range (IQR); categorical variables are presented as the relative frequency. We performed Chi-square tests, Fisher's exact tests, Student's *t*-

tests, and Mann Whitney *U* tests, when appropriate, to detect statistically significant differences between groups. To analyze treatment rates and predictors of treatment with DAAs, we excluded patients treated with IFN/RBV from the analysis. Patient follow-up was evaluated from January 2015 (when DAAs were introduced in Spain) until death or the end of the study, on September 30, 2019. Patient follow-up data were calculated in terms of person-years (p-y). Rates in p-y were defined as the quotient of the number of events observed during the study period (in the numerator) and the sum of all the individual follow-up times (in the denominator). We used Kaplan-Meier methods to estimate the cumulative incidence of treatment with DAAs. Cox regression models were used to analyze predictors of DAA treatment administration. All covariates that were significant in the univariate analysis were included in a multivariate analysis. *P* values < 0.05 were considered statistically significant. All statistical analyses were performed with Stata software (version 11.0; College Station, TX, United States).

RESULTS

Between October 2015 and September 2017, 501 patients (81.4% men) were enrolled in the OTP. The median age at study entry was 45 years (IQR: 39-50 years), 88% were Spanish-born and 96% of patients had been on opioid agonist therapy for more than 10 years (on average, since 2006; IQR: 2000-2014). The majority of patients (98.5%) was treated with methadone, 70% were unemployed, 49.5% had a history of incarceration and 65% had used injected drugs.

A total of 336 (67%) patients tested positive for anti-HCV antibodies (83% men; median age 46 years, IQR: 41-51 years); these patients had been taking opioid agonist therapy for a median of 15.3 years (IQR: 5.6-19.2 years). The prevalence of alcohol, cannabis, and cocaine use at study entry was 47.2%, 41.3%, and 31.9%, respectively. The prevalence of HIV co-infection was 47.6% (160/336). The characteristics of anti-HCV positive patients are shown in Table 1.

Of the 336 anti-HCV positive patients, 233 (69.3%) had positive results on an HCV-RNA test. The median of HCV-RNA was 11.4 (IQR: 1.5-44.8) × 10^5 IU/mL and the majority (59.2%) of cases were genotype *1a/1b*. Only 28 (8.3%) patients had cleared the infection, and 59/308 (19.1%) had been previously treated with IFN/RBV. HCV-RNA was not determined in 75 patients that were anti-HCV positive (22.3%). The distribution of patients according to HCV infection status is shown in Figure 1.

Rates and predictors of HCV treatment with DAAs

As of September 2019, among the 249 patients eligible (Figure 1) for DAA treatment, 111 (44.6%) were treated. Of those, 90% achieved SVR. The most frequent DAA combinations were sofosbuvir/ledipasvir, sofosbuvir/velpatasvir, and glecaprevir/pibrentasvir.

Rates and predictors of whether patients received DAA treatment

The 249 patients eligible for DAA treatment were followed-up for a median of 4.3 years (IQR: 2.4-4.7 years; total follow-up 879.3 p-y). The overall DAA treatment rate was 12.6/100 p-y (95%CI: 10.5-15.2) and treatment rates increased from 7.8/100 p-y (95%CI: 5.0-12.3), in 2015, to 18.9/100 p-y (95%CI: 11.7-30.3), in 2019.

Figure 2 shows treatment rates with DAAs since 2015. Patients with HCV-HIV co-infection had a treatment rate of 18.0/100 p-y (95%CI: 14.2-22.8); in contrast, patients with HCV mono-infection had a treatment rate of 8.6/100 p-y (95%CI: 6.4-11.7), (P < 0.001). Thus, the incidence rate ratio was 2.09 (95%CI: 1.4-3.1).

Figure 3 shows the Kaplan-Meier estimates of receiving treatment with DAAs. After four years, the probability of receiving DAA treatment was 39.5% (95CI%: 33.6-46.0) overall, 32% (95CI%: 24.4-41.0) in the HCV mono-infected patients, and 48.1% (95%CI: 39.3-57.8) in the HIV co-infected patients (P < 0.001).

The Cox regression models showed that HIV co-infected patients were twice as likely to receive HCV treatment, compared to those with HCV mono-infection (HR = 1.94, 95%CI: 1.21-3.12, P = 0.006). In addition, patients with ongoing drug use while in the OTP were 2.1-fold less likely to receive DAAs (HR = 0.48, 95%CI: 0.29-0.80) compared to those who do not used drugs (P = 0.004). The Cox regression models on predictors of treatment are shown in Table 2.

Table 1 Sociodemographics, substance use characteristics and blood parameters of anti-hepatitis C virus positive patients in an Opioid Treatment Program

	Anti-HCV positive, *n* = 336, *n* (%)
Female, *n* (%)	57 (17.0)
Age (yr), median (IQR)	46 (41-50)
Time in OTP (yr), median (IQR)	15.3 (5.6-19.2)
Opiate agonists	
Methadone	331 (98.5)
Buprenorphine	5 (1.5)
Antecedent of injection drug use (*n* = 326)	282 (86.5)
History of incarceration (*n* = 291)	158 (54.3)
Current substance use (last month) (*n* = 213), *n* (%)	
Alcohol	101 (47.2)
Cannabis	88 (41.3)
Cocaine	68 (31.9)
Heroin	49 (23.0)
Blood parameters	
Leucocyte ($\times 10^9$/L) (*n* = 298)	6.7 (5.3-8.6)
Lymphocyte ($\times 10^9$/L) (*n* = 296)	2.2 (1.5-2.8)
Platelets ($\times 10^9$/L) (*n* = 295)	181 (138-232)
Hemoglobin (mg/dL) (*n* = 296)	14.3 (13-15.1)
AST (U/L) (*n* = 284)	31 (21-52)
ALT (U/L) (*n* = 252)	30 (18-52.5)
GGT (U/L) (*n* = 242)	44.5 (25-89)
Total bilirubin (mg/dL) (*n* = 238)	0.5 (0.4-0.7)
Total cholesterol (mg/dL) (*n* = 210)	168.5 (144-194)
HIV infection, *n* (%) (*n* = 334)	160 (47.9)

HCV: Hepatitis C virus; IQR: Interquartile range; OTP: Opioid Treatment Program; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: Gamma glutamyl transferase; HIV: Human immunodeficiency virus.

DISCUSSION

This study provides a snapshot of the access to curative HCV treatment in patients treated with methadone. Furthermore, it shows that after the introduction of DAAs in Spain, nearly 50% of patients with an anti-HCV positive test were treatment naive. Moreover, we observed significantly lower rates of treatment among patients with HCV mono-infection than among patients with HCV-HIV co-infection.

Few studies in Spain have analyzed DAA treatment rates among patients enrolled in an OTP. In contrast, a European study showed that, after the introduction of DAAs, HCV treatment rates were 23/100 p-y among individuals that injected drugs and had HCV-HIV co-infection[23], which was twice the rate observed in our study. However, it is interesting to note that, since 2015, the proportion of patients that received treatments against HCV infection has increased and that DAA treatment showed efficacy in this difficult to treat population. In fact, the HCV treatment guidelines provided by the AASLD, EASL, and WHO have recommended individualized treatments for patients in the OTP[12-14].

In this study, HIV co-infection and ongoing drug use while in OTP were two independent predictors of whether a person received HCV treatment. The probability of being treated against infection was significantly higher in the co-infected group compared to the HCV mono-infection group. This finding might be related to

Table 2 Cox regression models for predictors of hepatitis C virus-treatment with direct antiviral agents

	Unadjusted HR (95%CI)	Adjusted HR (95%CI)
Female	0.79 (0.46-1.34)	
Age: 5 years increase	1.17 (1.06-1.30)	0.98 (0.81-1.18)
OTP and substance use related variables		
Time in OTP (yr)	1.03 (1.01-1.06)	1.02 (0.99-1.05)
Alcohol use (last month)	0.58 (0.37-0.92)	0.72 (0.45-1.17)
Substance use[1] (last month)	0.47 (0.30-0.74)	0.48 (0.29-0.80)
Antecedent of injection drug use	1.35 (0.72-2.51)	
History of incarceration	1.10 (0.75-1.63)	
Co-morbidity		
HIV infection	2.23 (1.52-3.28)	1.94 (1.21-3.12)

[1]Cannabis, cocaine, heroin. OTP: Opioid Treatment Program; HIV: Human immunodeficiency virus.

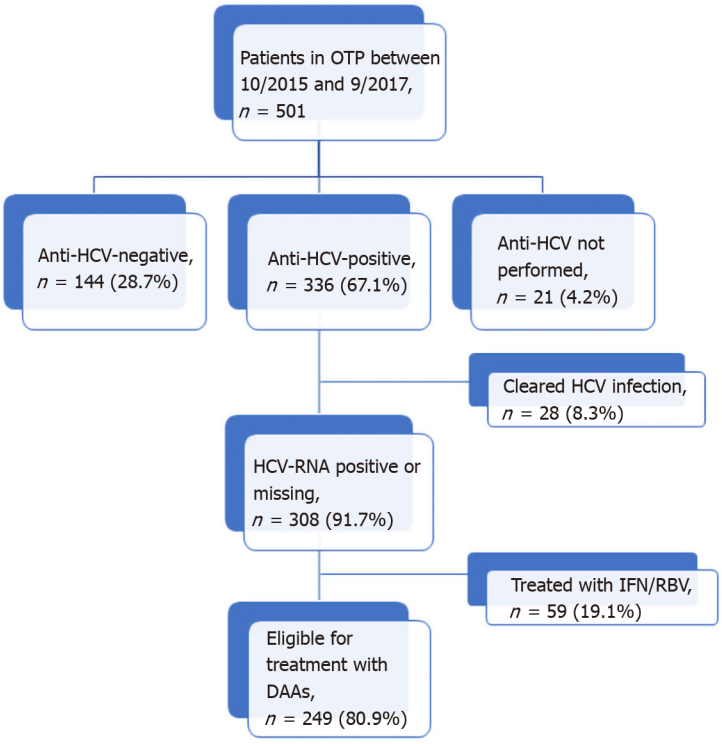

Figure 1 Flowchart of patients visited in the Opioid Treatment Program and hepatitis C virus infection status. OTP: Opioid Treatment Program; HCV: Hepatitis C virus; DAAs: Direct antiviral agents.

differences in the continuum of care in the HCV mono-infected and the HIV co-infected. In Spain, HCV mono-infected patients receive regular care and treatment in hospital-based Hepatology units while HCV-HIV co-infected patients are managed in HIV/Aids units having integrated services, psychosocial support and flexible time-slots for visits.

In this cohort, current drug use was associated with a lower probability of receiving HCV treatment. In this sense, health care professionals may perceive current drug use as a barrier to prescribe HCV treatment, despite international guidelines that recommend treatment of infection[12-14]. In patients with SUD, treating HCV infection has been considered a preventive intervention aimed to halt the transmission[24,25]. A recent clinical trial used electronic blisters to monitor adherence to DAA treatment

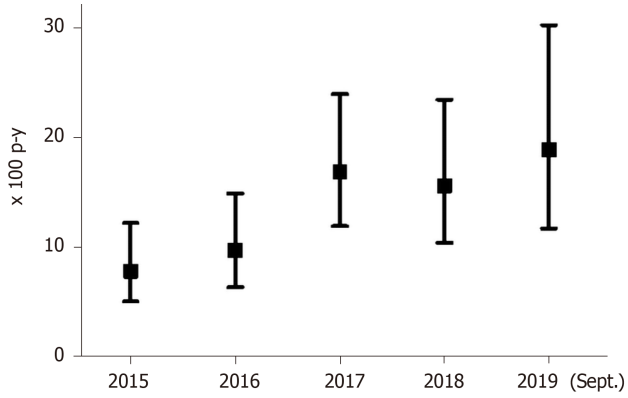

Figure 2 Annual rates of hepatitis C virus treatment with direct antiviral agents in an Opioid Treatment Program. p-y: Person-years.

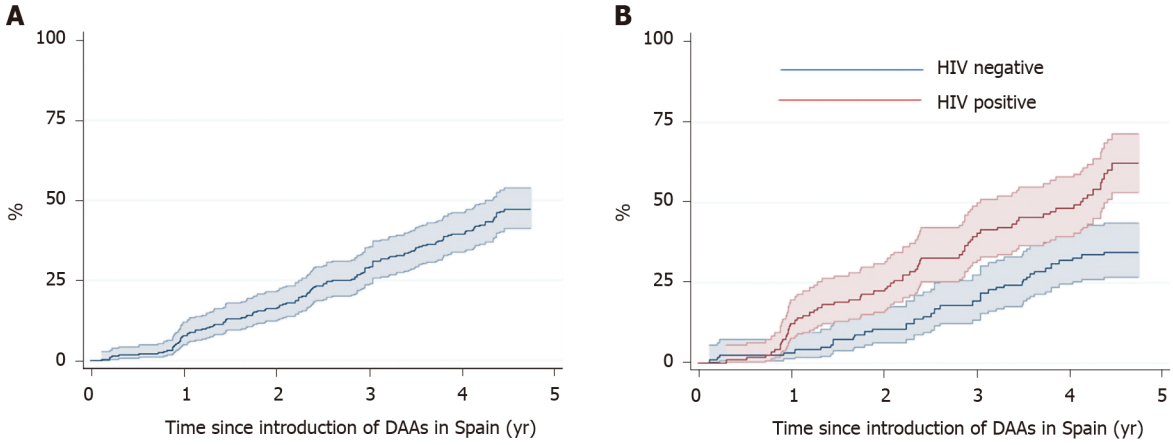

Figure 3 Kaplan Meier estimates (95%CI) of direct antiviral agent treatment for hepatitis C virus. Plots included (A) all patients and (B) patients with hepatitis C virus (HCV) mono-infection and HCV-human immunodeficiency virus co-infection. HIV: Human immunodeficiency virus; DAAs: Direct antiviral agents.

among patients that used drugs and were in an OTP; they showed that 97% of participants completed the treatment, and 94% achieved SVR[5].

This study had some limitations. First, the external validity of the results might have been limited due to the single-center study design. However, the OTP studied was the largest operating in metropolitan Barcelona, Spain, and only authorized to provide methadone or buprenorphine in a large urban area. Second, data related to the dose of methadone were not available and we also lacked data on treatment adherence and potential pharmacological interactions that might have led to DAA discontinuation. However, few studies have reported significant pharmacological interactions between DAAs and methadone[26,27]. Although some DAAs can increase the methadone or buprenorphine concentrations in blood, dose adjustments are not required, and monitoring withdrawal symptoms is merely recommended[27,28]. Third, we could have underestimated the HCV treatment rate with DAAs because some anti HCV-positive patients were considered treatment eligible without having a confirmatory RNA-HCV test.

In contrast, our study population is anchored in an OTP with a large number of patients and real-world conditions which is relevant to generate evidence in a population difficult to treat and retain.

CONCLUSION

In conclusion, this study highlights the challenges of measuring the continuum of HCV care while in an OTP. The goal of HCV elimination requires more targeted interventions to rapidly identifying those out of care.

ARTICLE HIGHLIGHTS

Research background

The introduction of direct-acting antiviral agents (DAAs) is associated with substantial changes in clinical outcomes of hepatitis C virus (HCV) infection. In this context, individuals with substance use disorder (SUD) have been recognized as a target population for the treatment of HCV infection.

Research motivation

Retention in treatment of SUD is key for the assessment and cure of HCV. In HCV infection, up to 80% of persons who inject drugs are infected but only a proportion is on treatment. In this sense, it is important to know real-life data in drug use populations. The Opioid Treatment Program (OTP) in metropolitan Barcelona, Spain, reports an increasing proportion of patients that are eligible for HCV treatment with DAAs.

Research objectives

Our main objective was to assess HCV infection status and treatment rates in a population primarily admitted for the treatment of SUD. Given the longitudinal nature of the study we aimed to identify gaps and challenges in using DAAs. In doing so we hypothesized on potential barriers that difficult the access to treatment in this population.

Research methods

We specifically analyzed annual treatment rates with DAAs in the context of HCV mono-infection and human immunodeficiency virus (HIV) co-infection. In addition, we estimated the cumulative incidence and main predictors of HCV treatment.

Research results

Results confirm a high prevalence of HCV infection in the OTP (67%) and the increasing rates of treatment over time. Almost 50% of HCV-positive patients were treatment naive (as of September 2019) in a health care system without restrictions in terms of insurance coverage. Patients with ongoing drug use and those with HCV mono-infection were less likely to be treated with respect to those with HIV co-infection.

Research conclusions

To the best of our knowledge this is the first study in Spain reporting on HCV treatment rates with DAAs in an OTP. We conclude that treatment rates increase over time and that higher rates are observed in the HIV-coinfected. The observed differences may be related to the lack of integrated care services for the HCV mono-infected. In addition, current drug use has an impact on the readiness to treat HCV infection.

Research perspectives

The goal of HCV elimination requires targeted interventions to identify those out of care and to implement strategies focused on traditional and local barriers. Surmounting barriers is necessary to eradicate HCV infection in people seeking treatment of SUD. The integrated management of liver disease with hepatologists, infectious diseases and addiction specialists may have an impact in reducing end stage liver disease.

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ORIGINAL ARTICLE

Observational Study

Comparative study between bowel ultrasound and magnetic resonance enterography among Egyptian inflammatory bowel disease patients

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Author contributions: Kamel S completed study design, bowel ultrasound examination, data collection and data analysis; Sakr M, Hamed W, Eltabbakh M and Askar S reviewed the manuscript for important scientific content; Bassuny A finished data collection; Hussein R reviewed the magnetic resonance imaging scans as an expert radiologist with high experience in inflammatory bowel disease, reviewed and edited radiological part of the manuscript; ElBaz A completed literature search, data analysis and writing the manuscript; all authors have read and approved final manuscript.

Institutional review board statement: The study was reviewed and approved by the Ain Shams University, Faculty of

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Abstract

BACKGROUND

Bowel ultrasound and magnetic resonance enterography (MRE) are decisive medical imaging modalities for diagnosing and locating bowel lesions with its extramural extent and complications. They assess the degree of activity, help clinicians to identify patients in need of surgery, and can be used for patient follow-up.

AIM

To compare the role of MRE and bowel ultrasound in diagnosis and follow-up of inflammatory bowel disease (IBD) patients in Egypt.

METHODS

The study was conducted on 40 patients with IBD. All patients were subjected to clinical assessment, laboratory investigations, bowel ultrasound, MRE, and colonoscopy up to the terminal ileum with biopsies for histopathological examination.

RESULTS

This study was conducted on 14 patients (35%) with ulcerative colitis and 26 patients (65%) with Crohn's disease; 34 (85%) of these patients had active disease. Bowel ultrasound detected different bowel lesions with the following accuracies: ileum (85%), large bowel (70%), fistula (95%), stricture and proximal dilatation (95%) and abscesses (100%). Also, it showed that statistically significance of bowel

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ultrasound in differentiation between remission and activity of IBD in comparison to MRE and colonoscopy.

CONCLUSION
In comparison to MRE, bowel ultrasound is a useful, non-invasive, and feasible bedside imaging tool for the detection of inflammation, detection of complications, and follow-up of IBD patients when performed by the attending physician.

Key Words: Bowel ultrasound; Colonoscopy; Crohn's disease; Magnetic resonance enterography; Ulcerative colitis; Inflammatory bowel disease

Core Tip: Crohn's disease and ulcerative colitis are chronic, relapsing inflammatory bowel diseases (IBD). Medical imaging is decisive for diagnosis bowel lesions with its complications. Magnetic resonance enterography (MRE) is one of these imaging techniques. Also, bowel ultrasound is becoming progressively important in management of IBD. Our aim of work in our study is to compare between the role of MRE and bowel ultrasound in diagnosis and follow up of Egyptian IBD patients.

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INTRODUCTION

Crohn's disease and ulcerative colitis are chronic, relapsing inflammatory bowel diseases (IBD). Medical imaging is decisive for diagnosis and locating the bowel lesion with its extramural extent and complications. It assesses its degree of activity, assigning patients in need for surgery and can be used for their follow-up.

Magnetic resonance enterography (MRE) is one specific imaging technique for such diseases[1]. T2W and T1W images after intravenous gadolinium have high accuracy for diagnosis and assessment of disease activity[2].

Bowel ultrasound is also becoming progressively important in IBD management. Ultrasonography is a noninvasive, non-radiating, cheap, and very available technique that is acceptable and tolerated by patients and can be used repeatedly for follow-up examinations. Ultrasonography for these patients needs higher frequency linear array probes (5-15 MHz) for assessment of the five-layer wall of the bowel[3].

Up until now, no previous comparative studies between bowel ultrasound and MRE for Egyptian patients who suffered/are suffering from IBD, either ulcerative colitis or Crohn's disease, have been published.

MATERIALS AND METHODS

Study population

Our study enrolled 40 patients who presented to our IBD center at Ain Shams University Hospital during the period from September 2017 to September 2018.

The study was approved by the medical ethics committee of Ain Shams University. The study population included adolescents who were over 18 years old. All patients provided written informed consent before enrollment. Patients were excluded if they had severe or uncontrolled comorbidities, such as cardio-respiratory, neurological, metabolic, liver, kidney diseases, claustrophobia, cardiac pacemaker, or implanted metal objects that prohibited use of MRE.

All patients reported a complete medical history, underwent thorough clinical examinations and laboratory investigations, including complete blood count, liver

profile tests, renal profile tests, C-reactive protein, erythrocyte sedimentation rate, MRE, bowel ultrasound, and colonoscopy up to the terminal ileum with biopsies for histopathological examination.

Clinical activity score for Crohn's disease was assessed by The Crohn's Disease Activity Index (CDAI). Clinical remission was determined if CDAI was < 150 points or no fistula drainage was found as assessed by the Fistula Drainage Assessment index[4]. Ulcerative colitis activity was assessed using the Truelove and Witts classification based on clinical and laboratory parameters, such as fever, frequency of bowel movements, rectal bleeding, tachycardia, anemia, and elevated erythrocyte sedimentation rate[5].

Colonoscopies were performed with a videoscope system from Olympus Exera II CV-180 after colonic preparation and fasting for six hours.

Bowel ultrasound

Bowel ultrasound was done by one examiner who had performed several previous general ultrasound examinations. This examiner was trained for several bowel ultrasound exams under supervision of an ultrasound gastroenterologist specialist at Sacco Hospital, Italy. Bowel ultrasound assessment was reviewed blindly compared to MRE and colonoscopy.

Patients were examined *via* ultrasound after a six-hour fasting period to minimize intestinal air contents. Examination was done by ultrasound machine (Toshiba Xario, Japan) with a low frequency curved-array transducer (2.5-4.5 MHz) to determine any pathological bowel motility or distension and any para-intestinal structures, such as abscesses, in all abdominal quadrants. Examination with a high-frequency linear-array transducer (6.0-8.4 MHz) was used for bowel wall examination starting with examination of the proximal colon followed with the distal one and then the small bowel[3]. This examination assessed criteria of inflammation such as thickness of bowel wall, inflammatory mesenteric fat and lymph nodes, hyperemia on color Doppler flow, and complications, such as stenosis, fistulas or inflammatory masses.

In the longitudinal direction, the bowel wall was measured for bowel thickness at its anterior wall or in an area in which it was more visible in order to avoid mucosal folds and haustrations. The cursor was placed at the end of the interface echo between the serosa and proper muscle to the start of the interface echo between the lumen and the mucosa[6,7].

Several criteria for stenosis diagnosis *via* ultrasound have been reported, such as thickened bowel wall, narrowing of the diameter of the lumen < 1 cm, hyperperistalsis of the pre-stenotic bowel, and proximal dilatation > 25-30 mm[8-10].

An abscess was indicated by bowel ultrasound as an irregular, avascular hypoechoic area with a small amount of internal echoes or air in the form of hyperechoic streaks[9].

MRE

The patient was instructed to have a low residue diet the day before the examination and was asked to fast at least 6 to 8 h before the onset of the procedure. Ingestion of 1 to 2 L of hyperosmolar oral contrast was performed for about 45 min before the magnetic resonance (MR) exam started. After full distension of the bowel, a spasmolytic medication was given to decrease bowel peristalsis to provide better bowel visualization. The examination was performed on 1.5-T MR machine, Achieva, Philips Medical System, Best, Netherlands in MRI Unit, Ain Shams University Hospital. The patient was laid in supine position using a multi-element phased array Torso coil (16 channels). A dedicated MR study was then performed as described in Table 1. Pixel-based apparent diffusion coefficient maps were generated on the off-line workstation (extended workspace ''EWS''), Pride software (Philips Medical Systems). Intravenous gadolinium contrast was given (0.2 mmol/kg body weight) in dynamic fashion obtaining three-dimensional enhanced T1 isotropic volume excitation (3D-eTHRIVE) coronal scans at 10 s, 20 s, 60 s, 70 s, and 90 s. The total MRE procedure took about 30 to 45 min.

The images were interpreted by a radiologist with 12 years of experience in abdominal imaging and who was also blinded to the clinical and colonoscopy examination results.

The MRE evaluated bowel wall thickening, mural edema, enlarged mesenteric lymph nodes, restricted diffusion, peri-enteric vascularization (comb sign), peri-enteric fluid, and presence of complications, such as abscesses or fistulas.

Table 1 Magnetic resonance enterography imaging protocol

Imaging sequence and plane	TR/TE	Slice thickness (mm)	Gap	Field of view (mm)	Matrix
Coronal T2 SSFSE	1200/115	6	1	375 × 375	268 × 234
Coronal SSFP	3.2/1.56	6	0	375 × 375	252 × 233
Axial T2WI	1200/115	7	1	375 × 336	268 × 208
Axial DWI	2743/65	7	1	375 × 302	124 × 100
3D-THRIVE	4/1.9	-	0	410 × 377	196 × 178
Axial post contrast fat-suppressed gradient-echo T1WI	3.8/1.8	-	0	375 × 314	196 × 157
Coronal post contrast fat-suppressed gradient-echo T1WI	4/1.9	-	0	410 × 314	196 × 178

TR: Repetition time; TE: Echo time; SSFSE: Single-shot fast spin-echo; SSFP: Single-shot free precision; 3D: Three dimensional.

Statistical analysis

Statistical analysis was performed using the SPSS software (22.0 version: SSPS Inc., Chicago, IL. United States). Description of quantitative variables was expressed in the form of mean ± standard deviation (mean ± SD) or median and inter-quartile range. A description of qualitative variables was expressed by frequency and percentage. Comparison of qualitative variables was carried out using the chi-square test. $P < 0.05$ was taken as significant. The sensitivity, specificity, overall correctness of prediction, and positive and negative predictive values were calculated. Correlations were calculated using Pearson's correlation coefficient. The receiver operating characteristic (ROC) curves and areas under the ROC (AUROC) curves were applied to evaluate the prognostic values (specificity and sensitivity).

RESULTS

The demographic profile, clinical and laboratory parameters are shown in Table 2. Most of the patients were middle-age females who usually presented with abdominal pain and diarrhea. The result indicated that 14 (35%) of our patients had ulcerative colitis, and 26 (65%) had Crohn's disease while 34 (85%) of them were inactive. Four (4%) of studied patients had pancolitis, and 18 (45%) of the studied cases had ileal lesions.

Table 3 indicates that the bowel ultrasound appeared to be a good predictor for detection of ileal affection with sensitivity, specificity, and diagnostic accuracy of 93.8%, 50%, and 85%, respectively. With respect to the large bowel, bowel ultrasound detected large bowel affection with sensitivity, specificity, and accuracy of 37.5%, 91.7%, and 70%, respectively. Also, bowel ultrasound was a good predictor for detection of thickness of affected segment with sensitivity, specificity, and accuracy of 83.3%, 50%, and 60% respectively. Also, bowel ultrasound was a good predictor for detection of fistulous track with sensitivity, specificity, and accuracy of 85.7%, 100%, and 95%, respectively, while sensitivity, specificity, and accuracy of 100%, 94.4%, and 95%, respectively, for detection of stricture and proximal dilatation were found. Abscess was detected by bowel ultrasound in six patients with high specificity, sensitivity, and accuracy (100%). Also, bowel ultrasound showed that no statistically significant differences between bowel ultrasound and disease activity index, which indicates that bowel ultrasound can differentiate between remission and active disease (Figure 1).

Table 4 indicates that no statistically significant difference among bowel ultrasound, MRE, and colonoscopy for detection of activity of the disease was noted, indicating that bowel ultrasound and MRE can differentiate between remission and active IBD (Figure 2).

Table 5 compares between clinical symptoms and imaging modalities bowel ultrasound and MRE. It indicates that bleeding per rectum is statistically significant in patients with strictures and proximal dilatation during assessment by bowel ultrasound, while diarrhea is statistically significant to the extent of the lesions when assessed by MRE.

Table 2 Demographic characteristics, laboratory and colonscopic findings of the total 40 studied cases

Demographic characteristics, laboratory and colonscopic findings	*n*
Age (yr)	33.50 ± 8.19
Gender (male/female)	16/24
Symptoms	
Diarrhea	14 (35%)
Diarrhea and bleeding	10 (25%)
Bleeding	4 (10%)
Abdominal pain	36 (90%)
Total leukocyte count (10^3/cmm)	7.32 ± 2.22
Hemoglobin (g/dL)	11.22 ± 1.86
Total bilirubin (mg/dL)	0.97 ± 0.14
Alanine aminotransferase (IU/L)	25.75 ± 10.49
Total protein (g/dL)	7.16 ± 0.73
Albumin (g/dL)	3.76 ± 0.44
Blood urea nitrogen (mg/dL)	22.10 ± 9.86
Creatinine (mg/dL)	0.87 ± 0.22
Serum sodium (mmol/L)	136.65 ± 6.19
Serum Potassium (mmol/L)	3.96 ± 0.55
C-reactive protein (mg/L)	28 (6–55)
Erythrocyte sedimentation rate (mm/h)	45 (31.5–60)
Colonscopic findings	
Opacity of mucosa	28 (70%)
Excess exudate	24 (60%)
Cobble stone	26 (65%)
Bleeding on touch	20 (50%)
Aphthous ulcers	30 (75%)
Diffuse ulceration	20 (50%)
Pseudopolyps	20 (50%)
Polyps	30 (75%)
Site of involvement	
Rectum	4 (10%)
Pancolitis	4 (10%)
Descending colon	4 (10%)
Rectum and sigmoid colon	10 (25%)
Ileum	18 (45%)
Type of disease	
Ulcerative colitis	14 (35%)
Crohn's disease	26 (65%)
Activity	
Remission	6 (15%)
Activity	34 (85%)

Data are mean ± standard deviation, *n* (%) and median (inter-quartile range).

Table 3 The diagnostic characteristics of the bowel ultrasound in the detection of small intestinal and large bowel disease and its correlation to disease activity index

	Bowel ultrasound					Disease activity index		
	Sensitivity	Specificity	PPV	NPV	Accuracy	Remission	Activity	*P* value
Large bowel	37.5%	91.7%	75%	68.8%	0.700	0 (0)	8 (23.5%)	0.184
Ileum	93.8%	50%	88.2%	66.7%	0.850	6 (100%)	28 (82.4%)	0.264
Thickness (> 3 mm)	83.3%	50%	41.7%	87.5%	0.600	4 (66.7%)	20 (58.8%)	0.718
Extent	33.3%	85.7%	50%	75%	0.700	0 (0)	8 (23.5%)	0.184
Mesenteric lymphadenopathy	16.7%	71.4%	20%	66.7%	0.550	0 (0)	10 (29.4%)	0.125
Fistula	85.7%	100%	100%	92.9%	0.950	0 (0)	12 (35.3%)	0.082
Stricture and proximal dilatation	100%	94.4%	66.7%	100%	0.950	0 (0)	6 (17.6%)	0.264
Abscess	100%	100%	100%	100%	0.1	0 (0)	6 (17.6%)	0.264

Data are *n* (%). PPV: Positive predictive value; NPV: Negative predictive value

Table 4 Comparison between bowel ultrasound, magnetic resonance enterography and colonoscopy as regards the detection of disease activity and remission

Activity	Colonoscopy	Bowel ultrasound	MRE	*P* value
Remission	6 (15%)	6 (15%)	6 (15%)	1.000
Activity	34 (85%)	34 (85%)	34 (85%)	1.000

Data are *n* (%). MRE: Magnetic resonance enterography.

DISCUSSION

Endoscopy is still the most important diagnostic procedure as it permits taking biopsy for histological examination[11]. European guidelines have recommended imaging techniques, such as bowel ultrasound, computed tomography enterography, and MRE as complementary tools for IBD diagnosis that can help define its location, extension, and complications[12].

MRE is a cross-sectional non-ionizing imaging technique that can be used for IBD diagnosis and extrainstestinal assessment of disease activity and followup of patients. But MRE is available at certain centers only and it takes long time during scanning with sedation in some cases such as children to avoid motion artefacts besides non-compliance to contrast intake and breath-hold technique[13].

Assessment of gastrointestinal tract in IBD patients by intestinal ultrasound was evolved nowadays due to development of ultrasound devices and rising skillfullness of their examiners as radiologists and gastroenterologists. Major parts of the small and large intestine can be easily examined by bowel ultrasound while proximal part of jejunum and the rectum may be difficult in their assessment due to overlying structures. Inspite of different advantages of bowel ultrasound as a rapid bedside, inexpensive and non-radiating tolerable test but its results are subjective to the examiner's expertise[14].

Our results showed similar sensitivity for detection of ileal IBD in comparison to one previous study (92.7%) but with lower specificity than this study (88.2%). Regarding colonic IBD, our results showed lower sensitivity than observed in this previous study (81.8%) but with similar specificity (95.3%), a finding which may be explained by interobserver variability between examiners[13]. This variability can explain why one study concluded that bowel ultrasound is more accurate for assessment of IBD patients if combined with colonoscopy[16].

Table 5 Comparison between clinical symptoms and imaging techniques; bowel ultrasound and magnetic resonance enterography

	Abdominal pain		*P* value	Bleeding per rectum		*P* value	Diarrhea		*P* value
	No, *n* (%)	Yes, *n* (%)		No, *n* (%)	Yes, *n* (%)		No, *n* (%)	Yes, *n* (%)	
Bowel ultrasound									
Large bowel	0 (0)	8 (22.2%)	0.292	8 (25.0%)	0 (0)	0.114	2 (10.0%)	6 (30.0%)	0.114
Ileum	4 (100.0%)	30 (83.3%)	0.376	26 (81.2%)	8 (100.0%)	0.184	18 (90.0%)	16 (80.0%)	0.376
Thickness (> 3 mm)	4 (100.0%)	20 (55.6%)	0.085	18 (56.2%)	6 (75.0%)	0.333	14 (70.0%)	10 (50.0%)	0.197
Extent	0 (0)	8 (22.2%)	0.292	8 (25.0%)	0 (0)	0.114	2 (10.0%)	6 (30.0%)	0.114
Lymphadenopathy	0 (0)	10 (27.8%)	0.224	8 (25.0%)	2 (25.0%)	1.000	4 (20.0%)	6 (30.0%)	0.465
Fistula	0 (0)	12 (33.3%)	0.168	8 (25.0%)	4 (50.0%)	0.168	4 (20.0%)	8 (40.0%)	0.168
Stricture and proximal dilatation	0 (0)	2 (5.6%)	0.629	0 (0)	2 (25.0%)	0.004	0 (0)	2 (10.0%)	0.147
Abscess	2 (50.0%)	6 (16.7%)	0.114	6 (18.8%)	2 (25.0%)	0.693	6 (30.0%)	2 (10.0%)	0.114
MRE									
Large bowel	0 (0)	16 (44.4%)	0.085	14 (43.8%)	2 (25.0%)	0.333	6 (30.0%)	10 (50.0%)	0.197
Ileum	4 (100.0%)	28 (77.8%)	0.292	24 (75.0%)	8 (100.0%)	0.114	16 (80.0%)	16 (80.0%)	1.000
Thickness (> 3 mm)	2 (50.0%)	10 (27.8%)	0.358	10 (31.2%)	2 (25.0%)	0.730	6 (30.0%)	6 (30.0%)	1.000
Extent	0 (0)	12 (33.3%)	0.168	10 (31.2%)	2 (25.0%)	0.730	2 (10.0%)	10 (50.0%)	0.006
Lymphadenopathy	0 (0)	12 (33.3%)	0.168	8 (25.0%)	4 (50.0%)	0.168	8 (40.0%)	4 (20.0%)	0.168
Fistula	0 (0)	14 (38.9%)	0.122	10 (31.2%)	4 (50.0%)	0.320	6 (30.0%)	8 (40.0%)	0.507
Stricture and proximal dilatation	0 (0)	4 (11.1%)	0.482	4 (12.5%)	0 (0)	0.292	2 (10.0%)	2 (10.0%)	1.000
Abscess	0 (0)	6 (16.7%)	0.376	6 (18.8%)	0 (0)	0.184	2 (10.0%)	4 (20.0%)	0.376

MRE: Magnetic resonance enterography.

A bowel wall thickness cutoff value of 3 mm in our study showed sensitivity (83.3%), specificity (50%), and accuracy (60%) in comparison to other studies, which showed sensitivities of 88% to 94%; however, specificity (93%-97%,) and diagnostic accuracy (94%) were higher in previous studies than in our study[9,15]. This finding can be explained by the lack of international agreement about standardized measurement parameters, which leads to interobserver variability between examiners[18].

Mesenteric lymph nodes detected by bowel ultrasound in our study were non-sensitive and non-specific (16.7% and 71.4%, respectively) and insignificantly subsided during remission; therefore, lymph nodes detection was not a good parameter of activity in agreement with some previous studies[19,20].

Our study agreed with different trials in which it was shown that detection rate of fistulas, depending on their localization, had sensitivity between 67% and 82% and specificity between 90% and 100%[8,9,21-23].

Stricture in our study as detected by bowel ultrasound showed sensitivity, specificity, and accuracy (100%, 94.4%, and 95%, respectively) were similar to other studies[15,24-26].

The sensitivity for detecting abscesses in different studies varied between 80% and 100%, and specificity varied between 92% and 94%, which were similar results to

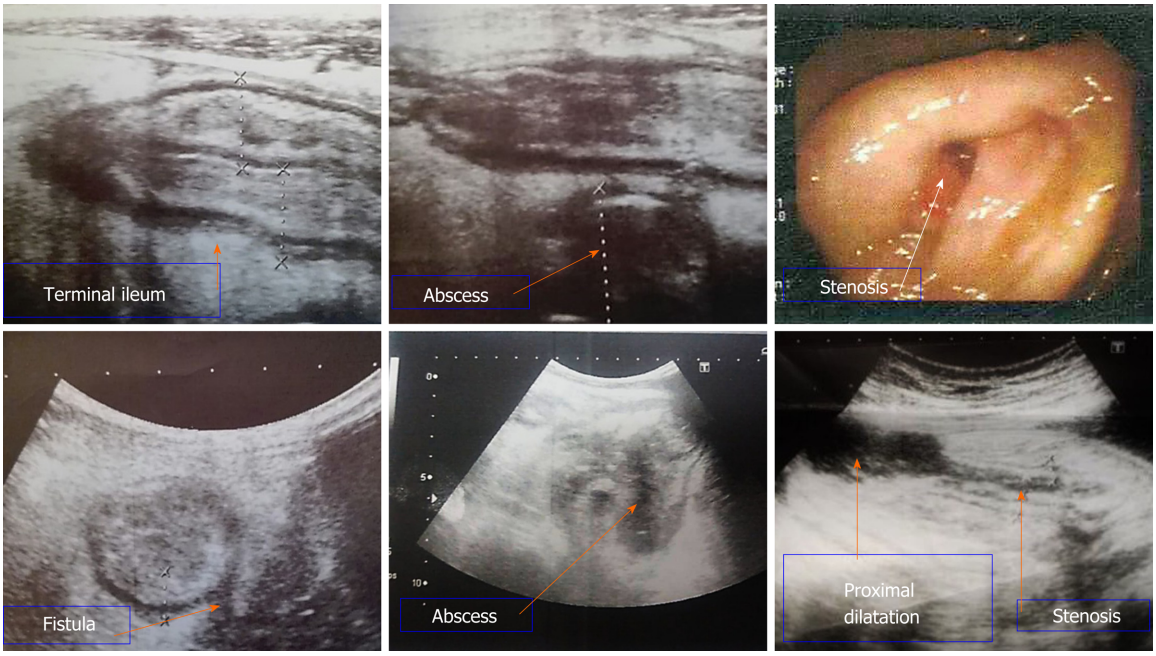

Figure 1 Bowel ultrasound and colonoscopy images. Bowel ultrasound demonstrates diffuse terminal ileal wall thickening likely of inflammatory nature with sonographic evidence of fistulization with mesenteric abscess formation. Stenosis which was detected during colonoscopy was seen by bowel ultrasound with proximal dilatation.

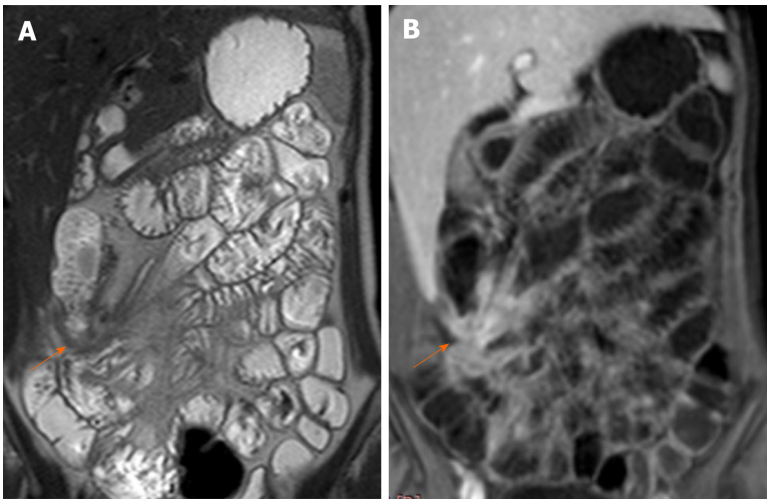

Figure 2 Magnetic resonance enterography. A: Coronal T2WI shows enteroenteric fistula at the right iliac fossa with stellate appearance of the thickened ileal loops (white arrow); B: Coronal fat-suppressed three-dimensional gradient echo postcontrast T1WI shows accentuated star-like enhancement at the right iliac fossa denoting fistulizing crohn's disease.

ours[27-29].

Our study agreed with different trials in which it was shown that diagnosis of IBD and assessment of its activity cannot be dependent on clinical evaluation alone but should by combined with other investigations such as biomarkers, endoscopy, and imaging techniques such as bowel ultrasound and MRE[25,30].

Our results showed that aphthous ulcers at endoscopy, stricture and mesenteric lymphadenopathy at bowel ultrasound, thickness of bowel wall and proximal dilatation at MRE were significantly correlated to disease activity (Figure 3). Other studies showed that other different bowel ultrasound parameters such as bowel wall thickening and its extent showed a significant correlation with disease activity[26,31,32]. Regarding MRE results in our study agreed with some studies[33,34] and disagree with another[35].

This indicates that there is no clear gold standard imaging technique for IBD diagnosis including MRE or bowel ultrasound which could be used besides clinical

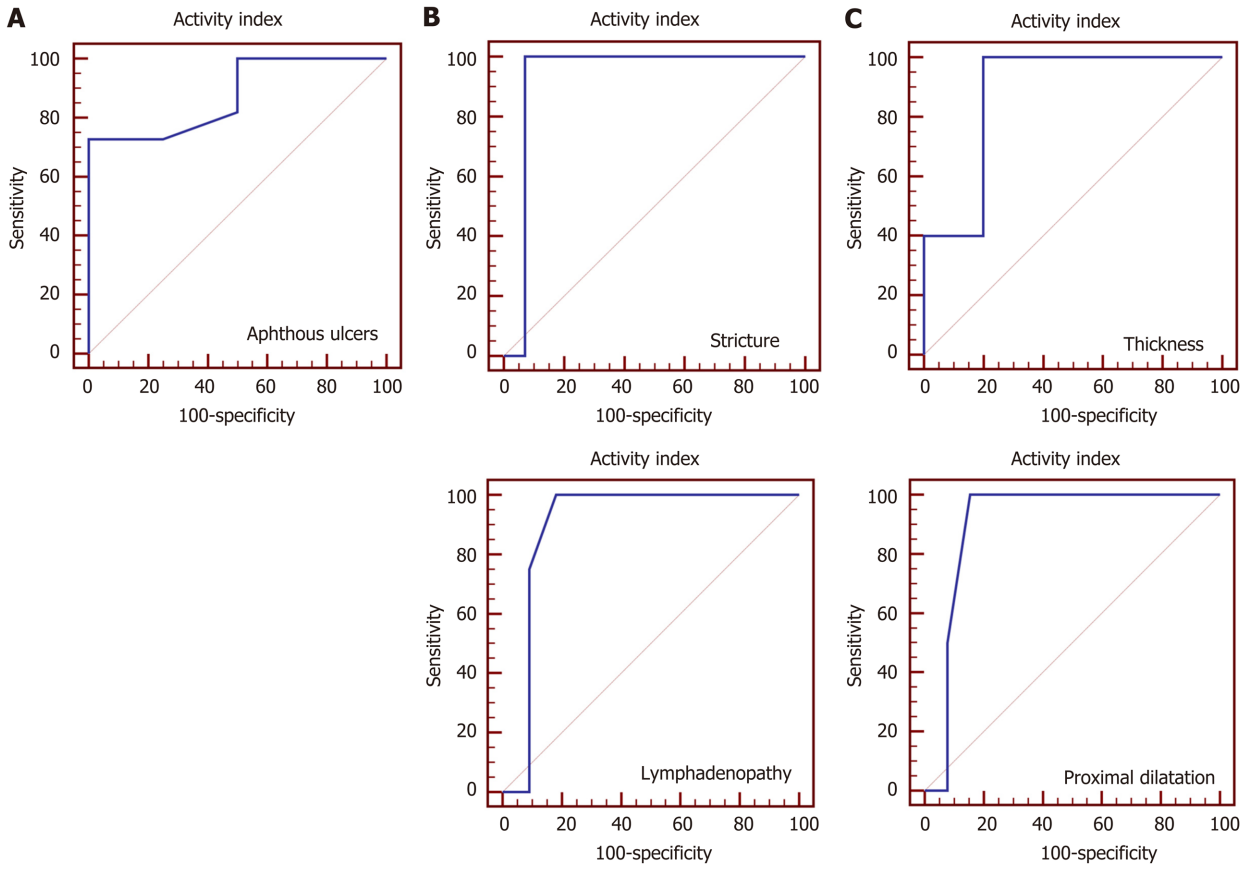

Figure 3 Receiver operating characteristic curve for prediction of active disease. A: At endoscopy, aphthous ulcers mean area under the receiver operating characteristic (ROC) curve was 0.875 ($P < 0.001$), positive likelihood ratio infinity, and negative likelihood ratio 0.28; B: Bowel ultrasound showed stricture and lymphadenopathy mean area under the ROC curve were 0.929 ($P = 0.036$) and 0.898 ($P = 0.01$) respectively, positive likelihood ratio infinity for both, and negative likelihood ratio 0.94 and 0.71 respectively; C: Magnetic resonance enterography showed thickness and proximal dilatation mean area under the ROC curve were 0.880 ($P < 0.001$) and 0.904 ($P = 0.033$) respectively, positive likelihood ratio 1.06 and infinity respectively, and negative likelihood ratio 0.88 for both.

history, biomarkers, endoscopy for diagnosis of IBD as agreed with previous studies[25,30,36].

Bowel ultrasound can be more helpful in follow-up of IBD patients and monitoring of their response to treatment away from its role of diagnosis by ultrasound guided biopsy.

In Egypt, both MRE and colonoscopy are available tools with estimated total cost of $93 dollars and $125 dollars respectively. Bowel ultrasound costs only 18$ dollars which is considered as a low cost alternative and has prospects for widespread clinical use.

Limitations of our study were the relatively small number of included patients, and comparative assessments of clinical decisions with and without bowel ultrasound were not available.

CONCLUSION

In comparison to MRE and colonoscopy, bowel ultrasound is a useful non-invasive and feasible bedside imaging tool for the detection of inflammation, complications, as screening tool and follow-up of IBD patients when performed by the attending physician.

ARTICLE HIGHLIGHTS

Research background

Bowel ultrasound is a new tool for evaluation of inflammatory bowel.

Research motivation
Up until now, no previous published comparative studies between bowel ultrasound and magnetic resonance enterography (MRE) for Egyptian inflammatory bowel disease (IBD) patients.

Research objectives
Compare between the role of bowel ultrasound and MRE in Egyptian IBD patients.

Research methods
The study was conducted on 40 patients presented to IBD center of Ainshams University Hospitals. The patients were subjected to clinical, laboratory, colonoscopic and radiological assessments including bowel ultrasound and MRE.

Research results
Bowel ultrasound was a good predictor of disease activity, fistula, stricture, and abscess formation with high sensitivity in ileum and more specificity in large bowel.

Research conclusions
Bowel ultrasound is a useful bedside cheap imaging tool that can be used for diagnosis and follow-up of IBD patients.

Research perspectives
Further studies to compare clinical decisions with and without bowel ultrasound.

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META-ANALYSIS

Tacrolimus and mycophenolate mofetil as second-line treatment in autoimmune hepatitis: Is the evidence of sufficient quality to develop recommendations?

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Abstract

BACKGROUND

The standard management of autoimmune hepatitis (AIH) is based on corticosteroids, alone or in combination with azathioprine. Second-line treatments are needed for patients who have refractory disease. However, high-quality data on the alternative management of AIH are scarce.

AIM

To evaluate the efficacy and safety of tacrolimus and mycophenolate mofetil (MMF) and the quality of evidence by using the Grading of Recommendations Assessment, Development and Evaluation approach (GRADE).

METHODS

A systematic review and meta-analysis of the available data were performed. We calculated pooled event rates for three outcome measures: Biochemical remission, adverse events, and mortality, with their corresponding 95% confidence intervals (CI).

RESULTS

The pooled biochemical remission rate was 68.9% (95%CI: 60.4-76.2) for tacrolimus, and 59.6% (95%CI: 54.8-64.2) for MMF, and rates of adverse events were 25.5% (95%CI: 12.4-45.3) for tacrolimus and 24.1% (95%CI: 15.4-35.7) for MMF. The pooled mortality rate was estimated at 11.5% (95%CI: 7.1-18.1) for

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tacrolimus and 9.01% (95%CI: 6.2-12.8) for MMF. Pooled biochemical remission rates for tacrolimus and MMF in patients with intolerance to standard therapy were 56.6% (CI: 43.4-56.6) *vs* 73.5% (CI: 58.1-84.7), and among non-responders were 59.1% (CI: 48.7-68.8) *vs* 40.8% (CI: 32.3-50.0), respectively. Moreover, the overall quality assessments using GRADE proved to be very low for all our outcomes in both treatment groups.

CONCLUSION
Tacrolimus and MMF are in practice considered effective for patients with AIH who are non-responders or intolerant to first-line treatment, but we found no high-quality evidence to support this statement.

Key Words: Autoimmune hepatitis; Efficacy; Grading of Recommendations Assessment, Development and Evaluation approach; Systematic review; Meta-analysis; Second-line

Core Tip: There is no consensus in the literature on which second-line treatment is superior in autoimmune hepatitis (AIH). This is the first systematic review and meta-analysis to compare the efficacy and safety of tacrolimus and mycophenolate mofetil (MMF) as second-line treatments in AIH. We also evaluated the quality of evidence for adding to the clinical guidelines for routine practice. We conclude that tacrolimus and MMF are considered effective for patients who are non-responders or intolerant to first-line treatment, but the quality of evidence is not high and it is questionable if these results should be added to clinical guidelines for AIH.

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INTRODUCTION

Autoimmune hepatitis (AIH) is a rare, chronic, inflammatory liver disease, characterized by elevated transaminase and immunoglobulin G levels, positive autoantibodies and interface hepatitis at liver histology[1,2]. It affects people of all ages and can lead to cirrhosis, hepatic failure, liver transplantation, and death[3]. Despite the availability of effective treatment and an evident good response to therapy, patients have a poor prognosis if the disease is left untreated or is treated suboptimally[4,5].

Standard first-line treatment of AIH is based on prednisolone, either given alone or in combination with azathioprine (AZA)[6]; these treatments lead to remission in 80% of patients[7,8]. However, about 20% of patients are refractory to standard treatment; this could be a result of suboptimal response, including treatment failure or incomplete response, or because patients are intolerant to standard treatment due to side-effects[8]. Thus, several second-line treatment modalities have been introduced for refractory AIH patients, including tacrolimus, mycophenolate mofetil (MMF), cyclosporine and budesonide[6-9]. Tacrolimus is a calcineurin inhibitor, which exerts more potent immunosuppressive effects on CD4+ T helper cells with fewer cosmetic side-effects. MMF is a purine antagonist similar to AZA, but it has more potent immunosuppressive properties and is better tolerated than AZA[10,11]. Tacrolimus and MMF have empirically been used the most, as alternative medications based on the American Association for the Study of Liver Diseases (AASLD) practice[8]. They are the most used drugs based on expert opinions[12]. Nevertheless, the efficacy and superiority of these interventions compared to using high-dose prednisolone and AZA has not been reported[8]. Furthermore, there is a lack of primary consensus on overall drug side-effects and disease mortality, at least for tacrolimus.

The accumulating but still sparse data indicate that refractory AIH patients do respond to these alternative treatments[13]. However, there is no firm evidence of their

effectiveness. First, there has been no randomized clinical trial (RCT) directly comparing these two medications to each other. Second, the available data are mainly based on small series of patients or case reports, from only a few centers, and little quality assessment has been performed. Thus, the translation of these findings to a guideline is questionable[14]. Third, two recent meta-analyses, which summarized the effect of the two medications in AIH, had a number of shortcomings. One study focused on improvement of aminotransferases rather than biochemical remission[15], while the second study included some (n = 12), but not all of the previously published studies related to MMF, and had analytical issues, such as reporting incorrect heterogeneity, ignoring existing publication bias, and reporting an incorrect overall mortality rate[16]. Therefore, their conclusions did not appear to be well supported by their results.

Furthermore, there is no systematic review or meta-analysis to compare tacrolimus with MMF as a second-line treatment in refractory AIH, or to evaluate the adverse effects, safety profile, and mortality rate of tacrolimus as a second-line treatment in refractory AIH. Recently two studies reported on the efficacy of tacrolimus[17] and MMF[18] as second-line treatment in AIH; these both need to be added to the overall assessment of the efficacy of these two medications in refractory AIH. In the absence of classical RCTs, and the shortcomings of previous investigations, there were still no comprehensive studies to evaluate the superiority of these two drugs for refractory AIH patients. The main question is whether there is sufficient high-quality scientific evidence to adapt the clinical guidelines.

Therefore, to study whether tacrolimus and MMF are superior alternative treatments, we firstly performed a systematic review and meta-analysis on the efficacy and safety of these treatments as second-line treatment in AIH patients. We also critically checked whether the quality of evidence, assessed by the Grading of Recommendations Assessment, Development and Evaluation (GRADE) approach[19,20], was sufficient to support adapting the clinical guidelines for routine practice.

MATERIALS AND METHODS

Protocol and registration

This study was conducted according to the guidelines in Meta-analysis of Observational Studies in Epidemiology (MOOSE)[21].

Information sources and search strategies

We reviewed the literature, focusing on our aim to identify, appraise, select and synthesize all the high-quality evidence available. To identify published articles and ongoing studies, we conducted a comprehensive search of electronic databases, including PubMed, EMBASE and Cochrane Central Register, with additional searching of the clinical trials website at www.clinicaltrials.gov. All databases were searched from their inception through October 2019, with no language restrictions. The search strategy was designed with the help of an experienced medical librarian and with input from investigators. Search terms were selected using Medical Subject Headings (Mesh) terms, including (but not limited to) "Hepatitis, Autoimmune", "Tacrolimus", "Mycophenolic Acid" and "Drug Resistance". In addition, to minimize the chance of missing any study, the reference lists of the included articles were searched individually for additional studies.

Eligibility criteria

We included studies which met the following criteria: Randomized or non-randomized controlled trials, case series of any duration, cohort studies, and reports that provided data on patients over 18 years with AIH who failed or were unable to tolerate first-line therapy prior to liver transplantation. Studies that used tacrolimus and/or MMF had to report on the disease outcomes studied. We excluded studies that reported data for AIH in children or adolescents aged ≤ 18 years, because they have a more aggressive disease, often with a more acute presentation[22] and they therefore need a different management[23]; or that reported data on patients who had had a liver transplantation.

Outcomes

Biochemical remission was the primary outcome for our study. This was defined as the disappearance of symptoms, normal serum bilirubin, γ-globulin and serum

aminotransferase levels[8]. Secondary outcomes were the occurrence of adverse events and mortality. We evaluated the available data for these outcomes per individual in the studies included in our analysis. For example, if γ-globulin levels were not described in a certain study, we used the variables available, such as clinical and biochemical variables, to reach the most likely definition of remission for that study.

Some studies reported biochemical remission depending on the reason for using second-line therapy (intolerance *vs* non-response). Thus, we performed a subgroup analysis in non-responders and those intolerant to standard therapy to compare the effect of tacrolimus and MMF in the two different groups that used the drug as second-line therapy.

Study selection and data extraction

After excluding duplicated reports, the reports included in our analysis were reviewed on the basis of their title and abstract by two independent reviewers (MA, MG). Thereafter, the full texts of selected reports were retrieved and independently assessed by both reviewers to identify which studies satisfied our inclusion criteria. Discrepancies between the two reviewers regarding eligibility were solved by jointly looking at the study in question. If no consensus was reached, a third reviewer (BZA) was consulted. Agreement was measured using inter-rater reliability (Cohen's Kappa).

Next, we extracted data on each study: Study characteristics (including the surname of the first author and year of publication), patients' characteristics (including mean age and number of patients), intervention characteristics (including duration of applied therapy and length of follow-up), as well as data on our primary and secondary outcome measures.

Methodological quality

The two reviewers independently rated the methodological quality of each study using the GRADE tool for study-level assessments of risk of bias[19,20]. Specifically, the domains assessed for risk of bias included: Failure to develop and apply appropriate eligibility criteria (inclusion of control population), flawed measurement of either intervention or outcome, failure to adequately control confounding, and incomplete follow-up.

GRADE quality assessment

The GRADE approach was used to assess the quality of evidence for primary and secondary outcomes[20,21]. It provides guidance for rating the quality of evidence and grading the strength of recommendations by asking a clear question for each outcome. The level of evidence was graded as high or moderate when it was derived from RCTs, and as low or very low if it was derived from observational studies. The level of evidence could be upgraded or downgraded depending on the quality of the study. The criteria provided for possibly downgrading the quality of evidence include: Risk of bias, publication bias, indirectness, imprecision and inconsistency. The three main reasons for upgrading the quality of evidence include: Large effect size, dose response gradient, and all plausible residual confounders increase confidence in the estimated effect. Each study can be given up to two points for every domain that begins with a high rating that is later downgraded one level (judged to have serious concerns) and two levels (if concerns are very serious). These evaluations result in one of four quality ratings – high, moderate, low and very low – that reveal the degree of confidence one can have in the available evidence correctly reflecting the theoretical true effect of the intervention. GRADE ratings are given as described by Balshem *et al*[24], and are adjudicated as high (we are very confident that the true effect lies close to that of the estimate of the effect), moderate (the true effect is likely to be close to the estimate of the effect, but there is a possibility that it could be substantially different), low (our confidence in the effect estimate is limited, *i.e.*, the true effect may be substantially different from the estimate of the effect), or very low (we have very little confidence in the effect estimate, *i.e.*, the true effect is likely to be substantially different from the estimate of effect).

Statistical analysis

Several studies reported the median, minimum and maximum values. Hence, in order to be able to combine the results, we deducted the sample mean and standard deviation (SD) using Wan *et al* 's[25] method for those studies. We estimated pooled event rates with corresponding 95% confidence intervals (CI) using the inverse variance method per analyzed outcome. We applied random effects models whenever there was significant heterogeneity between studies.

Q test, the associated *P* value ($P < 0.10$) and also the I^2 test were used to assess heterogeneity among the included studies. According to Higgins *et al*[26] $I^2 < 40\%$ indicates low heterogeneity, $30\% < I^2 < 60\%$ indicates moderate heterogeneity, while $50\% < I^2 < 90\%$ may represent substantial heterogeneity, and > 75% considerable heterogeneity. Publication bias was evaluated using funnel plots and Egger's test for asymmetry with 95%CI, with the results considered to indicate potential small study effects when $P < 0.10$[27]. Comprehensive meta-analysis software version 3.0 was used for our statistical analyses[28]. The statistical methods of this study were reviewed by Dr. Milada Småstuen Cvancarova from Oslo Metropolitan University, Oslo, Norway.

RESULTS

Study selection

The initial database search yielded 243 valid hits, of which 71 were duplicates and removed (Figure 1). The titles and abstracts of the remaining 172 citations were screened, leading to exclusion of a further 87 citations. The remaining 85 articles were assessed for relevance to our outcomes of interest. A full text review led to 64 articles being excluded. Overall, 21 studies met our eligibility criteria. Cohen's Kappa for inter-rater reliability between the two reviewers was 0.88. Thus, 21 unique observational studies[17,18,29-47] were eligible to be included in the meta-analysis and to be evaluated on the scientific quality of data using GRADE. The search results are summarized in Figure 1.

Study characteristics

Nine studies reported data on tacrolimus and 16 on MMF. Their geographical distribution was diverse, with studies carried out in the United States, Canada, United Kingdom, Denmark, Germany, the Netherlands, Belgium, Sweden, China, India and Australia. Most studies included middle-aged subjects who were predominantly female. In the tacrolimus studies, the average follow-up time was longer than in the MMF studies (51 mo *vs* 38 mo), patients were younger (mean age 37.8 years *vs* 42.8 years) and more females (73.3% *vs* 71.2%) were observed. Applied dosages varied between 2.0 and 6.0 mg daily of tacrolimus, and between 0.5 g and 2.0 g daily of MMF. The basic characteristics of the studies are presented in Table 1.

Methodological quality of the studies

For each of the 21 included studies, the methodological quality (risk of bias) was assessed and downgraded for the presence of bias using the GRADE risk of bias tool on a scale of 0 to -2. Four studies were rated as having no risk of bias (scored as 0), 16 studies had a serious risk of bias (scored as -1) and the remaining two had a very serious risk of bias (scored as -2). The details of the assessment of the methodological quality of the studies is reported in Table 2 and Figure 2-4.

Pooled prevalence of biochemical remission

In total, we included 584 patients with AIH who were unable to tolerate or respond to first-line therapy. In 157 patients treated with tacrolimus, the overall pooled prevalence of biochemical remission was 68.9% (95%CI: 60.4-76.2), and in 427 patients with MMF 59.6% (95%CI: 54.8-64.2) (Figure 3 and 4).

Significant moderate heterogeneity was found among the 21 studies in the meta-analysis for tacrolimus and MMF ($Q = 16.25$ *vs* 29.72, df = 8 *vs* 15, $P < 0.0001$), respectively. According to I^2 values for the tacrolimus and MMF groups, approximately 50.8% *vs* 49.5% respectively, the variability in effect estimates was due to the heterogeneity between the studies rather than a sampling error or chance. Overall quality assessments using GRADE were very low for biochemical remission in both intervention groups (Table 2).

Egger's regression test for tacrolimus (intercept = 0.02, 95%CI: -2.14 to 2.18; $P = 0.98$) and for MMF (intercept = -0.36, 95%CI: -1.77 to 1.06; $P = 0.59$) did not show statistically significant asymmetry of the funnel plots, suggesting that publication bias was unlikely in both intervention groups (Figure 5).

Overall, 15 studies specified response rates according to the reason for using tacrolimus or MMF. In patients with intolerance to standard therapy, the pooled biochemical remission rate for tacrolimus was 56.6% (CI: 43.4-56.6), and 73.5% (CI: 58.1-84.7) for MMF. Among non-responders 59.1% (CI: 48.7-68.8) did respond to tacrolimus, while 40.8% (CI: 32.3-50.0) responded to MMF.

Table 1 Baseline characteristics and summary of findings of the 21 studies included in our meta-analysis of second-line treatments for autoimmune hepatitis refractory patients

Ref.	Year	Country	Patients (*n*)	Female	Mean age	Design	Biochemical remission (%)	Follow-up	Mean dose[2]
Tacrolimus									
Zolfino *et al*[29]	2002	United Kingdom	5	3/5	28.4 ± 12.26	Retrospective	2/5 (40)	NR	2-4
Aqel *et al*[30]	2004	United States	11	10/11	63	Retrospective	10/11 (91)	16[1]	3.0
Chatur *et al*[31]	2005	Canada	3	NR	NR	Retrospective	0/3 (0)	26.5 (10–54)	2-4
Larsen *et al*[32]	2007	Denmark	9	8/9	36 ± 16.06	Retrospective	9/9 (100)	21.25 ± 8.37	2 (2-4)
Yeoman *et al*[33]	2011	United Kingdom	9	5/9	39.5 ± 18.07	Retrospective	7/9 (77.8)	NR	NR
Tannous *et al*[34]	2011	United States	13	10/13	40.6 ± 12.5	Retrospective	12/13 (92.3)	1-65	2-6
Than *et al*[35]	2016	German, United Kingdom	17	11/17	34.5 ± 15.03	Retrospective	9/17 (53)	84 ± 53.45	2 (0.5-5)[1]
Efe *et al*[36]	2017	Europe, United States, Canada, and China	80	60/80	34.7 ± 11.78	Retrospective	58/80 (72.5)	85.75 ± 37.83	3 (0-6)[1]
Pape *et al*[17]	2020	Belgium, Netherlands	10	8/10	38 ± 13.67	Retrospective	5/10 (50)	14.5 ± 6.47	3.5 ± 1.72
MMF									
Richardson *et al*[37]	2000	United Kingdom	7	6/7	27.28 ± 10.45	Retrospective	5/7 (71.4)	43 ± 13.92	2
Zolfino *et al*[29]	2002	United Kingdom	2	1/2	17.5 ± 2.12	Retrospective	0/2 (0)	NR	2
Devlin *et al*[38]	2004	Canada	5	4/5	54 ± 2.10.83	Retrospective	5/5 (100)	NR	1-2
Chatur *et al*[31]	2005	Canada	11	NR	NR	Retrospective	7/11 (63.6)	26.5 (10–54)	0.5-2
Czaja *et al*[39]	2005	United States	7	NR	NR	Retrospective	0/7 (0)	19 ± 7	0.5-3
Inductivo-Yu *et al*[40]	2007	United States	15	11/15	60 ± 15	Retrospective	11/15 (73.3)	41	2
Hlivko *et al*[41]	2008	United States	12	NR	NR	Retrospective	8/12 (66.7)	NR	0.5-2
Hennes *et al*[42]	2008	Germany	36	28/36	41.5 ± 13.24	Retrospective	14/36 (39)	35.38 ± 21.4	1.75 (0.5-3)[1]
Wolf *et al*[43]	2009	United States	16	NR	NR	Retrospective	12/16 (75)	NR	1-2
Sharzehi *et al*[44]	2010	United States	17	13/17	50	Retrospective	8/17 (48)	12	0.5-2
Yeoman *et al*[33]	2011	United Kingdom	2	1/2	31.5 ± 19.51	Retrospective	1/2 (50)	NR	NR
Baven-Pronk *et al*[45]	2011	The Netherlands	30	24/30	NR	Retrospective	14/30 (46.7)	39.5 ± 22.51	0.5-3
Jothimani *et al*[46]	2014	India, United Kingdom	19	16/19	52.25 ± 16.52	Retrospective	14 /19 (73.6)	45.4 ± 21.13	1-2
Roberts *et al*[47]	2018	Australia	105	92/105	52.5 ± 3.65	Retrospective	63/105 (60)	38.75 ± 10.14	2.0 (1.0-2.0)[1]
Efe *et al*[36]	2017	Europe, United States, Canada, and China	121	96/121	41.25 ± 13.45	Retrospective	84/121 (69.4)	66.25 ± 31.77	1 (0-2)[1]
Giannakopoulos *et al*[18]	2019	Sweden	22	12/22	50 ± 12.57	Retrospective	10/22 (45.5)	71 (10-54)[1]	2.0 (1.0–2.5)[1]

[1]Median.
[2]Mean dose in tacrolimus studies is defined as mg/d and in mycophenolate mofetil studies is defined as g/d. NR: Not reported; MMF: Mycophenolate mofetil.

Table 2 Summary of findings and quality assessment of evidence per our outcomes of interest using the Grading of Recommendations Assessment, Development and Evaluation approach

Outcomes	Certainty assessment							Patient (*n*)	Certainty	Importance
	Studies (*n*)	Study design	Risk of bias	Inconsis-tency	Indirect-ness	Imprecision	Other considerations	Per event/in total (%)		
Tacrolimus										
Biochemical remission	9	Observational	Serious[1]	Serious[2]	Not serious	Not serious	None	112/157 (71.3)	+OOO Very low	Critical
Adverse events	7	Observational	Serious[1]	Serious[2]	Not serious	Not serious	None	28/143 (19.6)	+OOO Very low	Important
Mortality	9	Observational	Serious[1]	Not serious	Not serious	Not serious	None	14/157 (8.9)	+OOO Very low	Critical
MMF										
Biochemical remission	16	Observational	Serious[1]	Serious[2]	Not serious	Not serious	Dose response gradient	256/427 (59.9)	+OOO Very low	Critical
Adverse events	13	Observational	Serious[1]	Serious[2]	Not serious	Not serious	None	97/416 (23.3)	+OOO Very low	Important
Mortality	16	Observational	Serious[1]	Not serious	Not serious	Not serious	Publication bias strongly suspected	22/427 (5.2)	+OOO Very low	Critical

[1]Failure to develop and apply appropriate eligibility criteria.
[2]Statistically significant heterogeneity. MMF: Mycophenolate mofetil.

Pooled event rate of adverse events

Frequencies and percentages of reported adverse events were not adequately mentioned in five studies (two studies in tacrolimus[29,33] and three studies in MMF[29,33,39]). Patients given tacrolimus had a number of adverse events, in which neurologic symptoms and gastrointestinal side-effects were the most common; 22 patients had to discontinue the drug due to adverse events. The most common adverse events associated with MMF were gastrointestinal side-effects and leukopenia, which led to 46 patients discontinuing the drug.

The pooled adverse event rate for tacrolimus was 25.5% (95%CI: 12.4-45.3) and for MMF was 24.1% (95%CI: 15.4-35.7) (Supplementary Figures 1 and 5). There was substantial significant heterogeneity among the studies in both intervention groups yield an I^2 = 66.73% (P_{het} = 0.006) for tacrolimus and 74.24% (P_{het} = 0.001) for MMF. Overall quality assessments using GRADE were very low for adverse events in both intervention groups (Table 2).

Egger's regression test for tacrolimus (intercept = 0.87, 95%CI: -3.35 to 5.09; P = 0.62) and for MMF (intercept = -0.36, 95%CI: -2.93 to 2.22, P = 0.77) did not show statistically significant asymmetry of the funnel plot, suggesting that publication bias was unlikely (Supplementary Figures 2 and 6).

Pooled mortality rate

The studies reported 14 deaths occurring in a total of 157 patients (8.9%) treated with tacrolimus and 22 deaths in 427 patients (5.2%) treated with MMF. The pooled mortality rate was 11.5% (95%CI: 7.1-18.1) in the tacrolimus group and 9% (95%CI: 6.2-12.8) in the MMF group (Supplementary Figures 3 and 7). There was no significant heterogeneity between studies for all-cause mortality in either intervention group, yielding an I^2 = 0% (P_{het} = 0.71) for tacrolimus and 12.46% (P_{het} = 0.31) for MMF. Overall quality assessments using GRADE were very low for mortality in both intervention

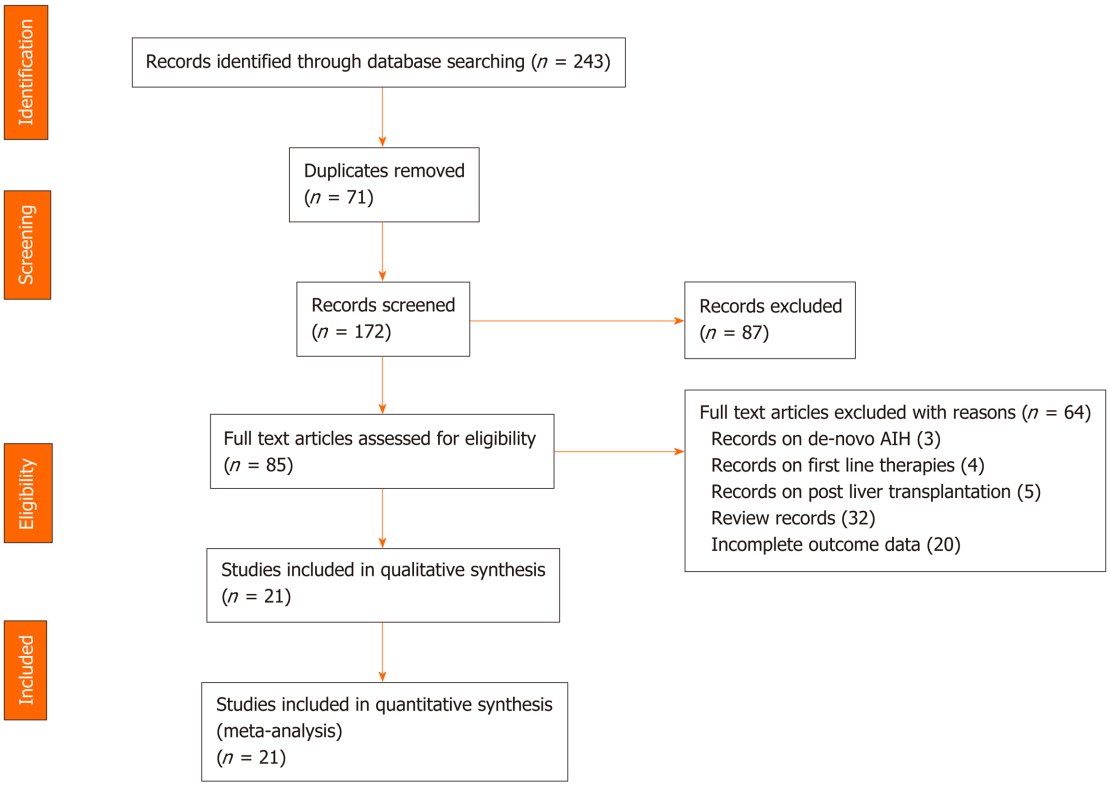

Figure 1 PRISMA flow diagram of the articles retrieved by systematic literature search. AIH: Autoimmune hepatitis.

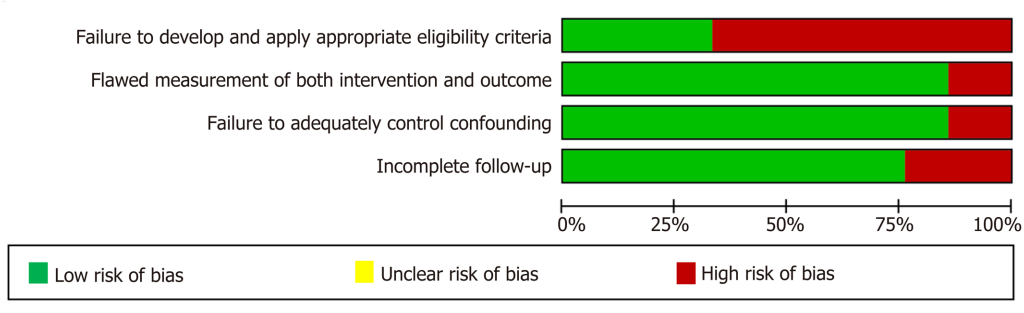

Figure 2 Assessment of methodological quality (risk of bias) of articles identified in the systematic literature search and included in our meta-analysis.

groups (Table 2).

Egger's regression test for tacrolimus (intercept = -0.35, 95%CI: -1.63 to 0.93, P = 0.54) did not show statistically significant asymmetry of the funnel plot, suggesting that publication bias was unlikely. In the MMF group (intercept = -1.08, 95%CI: -1.86 to -0.30, P = 0.01) there was statistically significant asymmetry of the funnel plot, suggesting there was a publication bias here (Supplementary Figures 4 and 8).

GRADE quality assessment of evidence

The GRADE quality scoring across the studies per outcome is summarized in Table 2. We rated the overall quality of the evidence to be very low for biochemical remission, due to inconsistency (statistically significant heterogeneity) and study limitations (risk of bias in 17 studies); as very low for adverse events due to inconsistency (statistically significant heterogeneity) and study limitations (risk of bias in 14 studies); and as very low for mortality owing to study limitations (risk of bias in 17 studies).

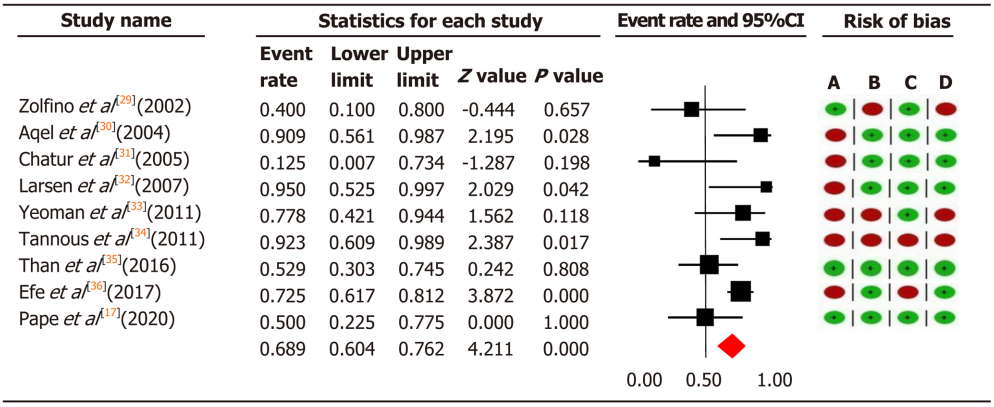

Figure 3 The pooled event rate of biochemical remission in the tacrolimus group with risk of bias assessment per study. Heterogeneity: Q = 16.25, degree of freedom = 8 (P = 0.039); I^2 = 50.76%. Test for overall effect: Z = 4.21 (P < 0.0001). A: Failure to develop and apply appropriate eligibility criteria (inclusion of control population); B: Flawed measurement of both exposure and outcome; C: Failure to adequately control confounding; D: Incomplete follow-up. CI: Confidence interval.

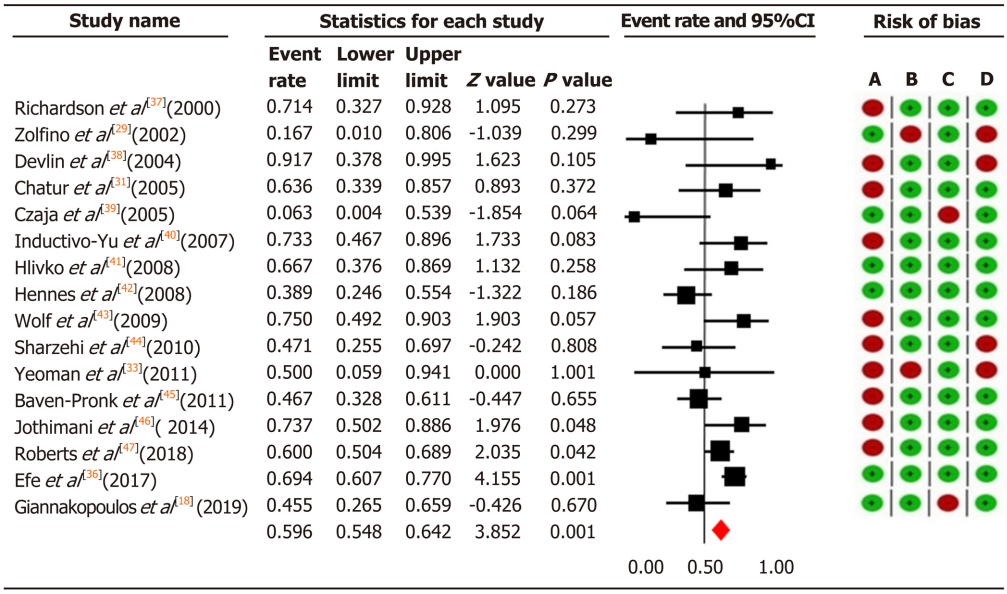

Figure 4 The pooled event rate of biochemical remission in the mycophenolate mofetil group with risk of bias assessment per study. Heterogeneity: Q = 29.72, degree of freedom = 15 (P = 0.013); I^2 = 49.52%. Test for overall effect: Z = 3.85 (P = 0 < 0.0001). A: Failure to develop and apply appropriate eligibility criteria (inclusion of control population); B: Flawed measurement of both exposure and outcome; C: Failure to adequately control confounding; D: Incomplete follow-up. CI: Confidence interval.

DISCUSSION

In our comprehensive analysis of 21 observational studies, comprising a total of 584 patients, we first evaluated the efficacy and safety of tacrolimus and MMF as a second-line treatment for patients with AIH. Two of our key findings are that tacrolimus is efficient in treating patients who did not respond to first-line treatments, yielding a biochemical remission rate of 59.1%, while MMF is considered effective for patients who are intolerant to the first-line therapy, yielding a biochemical remission rate of 73.5%.

In our critical assessment of the quality of the evidence using the GRADE approach, and in the absence of RCTs, we graded the quality of the observational studies as poor both for tacrolimus and MMF. Using the current evidence to develop therapeutic guidelines for these two medicines is therefore questionable. The three major strengths of our study are: The comprehensive search performed to trace all the eligible studies, our use of the rigorous methods given by the Cochrane Collaboration for data extraction, analysis and synthesis, and our assessment of risk of bias and the quality of

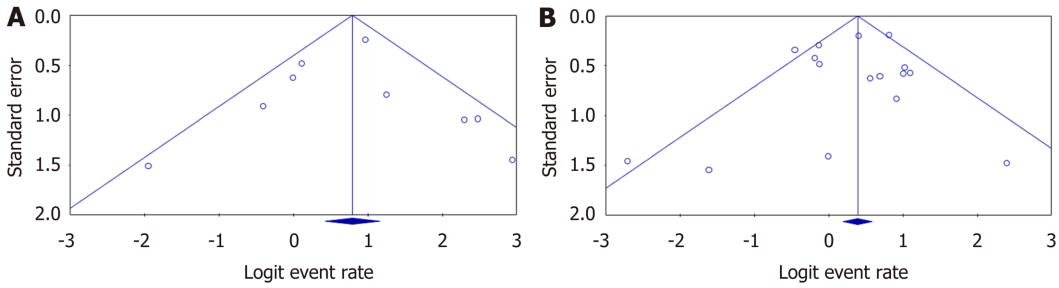

Figure 5 The publication bias of included studies for biochemical remission in (A) tacrolimus and (B) mycophenolate mofetil groups.

evidence using the GRADE approach.

One of the important aspects of an unbiased meta-analysis should be the performance of a comprehensive search for published studies. Previous meta-analyses[15,16] had several methodological problems, such as including studies on the treatment of naive AIH patients[15], not including all previously published studies[16], statistical errors such as reporting incorrect heterogeneity, not reporting on publication bias, and incorrect mortality rate[16]. Thus, their estimates on the efficacy of tacrolimus and MMF as second-line treatment modalities for AIH may not be accurate, and their conclusions on the superiority of these drugs may not be truly supported by their results. In the light of these shortcomings, we decided to perform a systematic review and meta-analysis of all the reported studies to date.

Biochemical remission

We found tacrolimus and MMF to be efficient interventions in treating patients who were non-responders or intolerant to first-line treatment. In our meta-analysis, we found a 59.1% biochemical remission rate in patients who were non-responsive to first-line therapy. The results from previous studies varied widely: Some found tacrolimus to be completely effective on biochemical remission[30,32,34], whereas others reported low remission rates[29,31]. In three studies, tacrolimus had a biochemical remission rate of more than 90% in refractory AIH patients[30,32,34], while another study reported a remission rate of 77% in refractory AIH patients[33]. More recently, Than *et al*[35] reported that 53% of refractory AIH patients responded to tacrolimus.

Similarly, previous meta-analysis has focused on the improvement of aminotransferases rather than biochemical remission, and reported an average rate of improvement of 78.7%[15]. Our study suggests tacrolimus can improve biochemical remission in 68.9% of patients with refractory AIH who responded to tacrolimus as second-line therapy.

However, current data concerning the efficacy of MMF in patients intolerant to first-line therapy are inconclusive. We found a 73.5% biochemical remission rate for MMF taken by patients intolerant to first-line treatment. Our findings agree with those of previous studies[38,44,45] that state that biochemical remission is significantly more common in intolerant AIH patients compared to those who were non-responsive. Some studies found MMF to be effective in intolerant patients, whereas other studies reported biochemical remission rates of less than 25% in non-responders[39,42,44-46]. Likewise, a recent meta-analysis[16], although it had various limitations, found a pooled remission rate much greater (82%) in patients intolerant to standard therapy compared to non-responders (32%). The difference in MMF biochemical remission rates in patients intolerant to first-line treatment in our study compared to a previous meta-analysis[16] (73.5% *vs* 82%) is likely due to their incomplete or selective inclusion of published studies. Given these data, MMF does seem to be a useful alternative therapy for AIH patients who are intolerant to first-line treatment.

Safety and side-effects

Tacrolimus: There are few reports of side-effects of tacrolimus in AIH patients. To date, our study is the first to evaluate the pooled adverse event rate and safety profile of tacrolimus as a second-line treatment for refractory AIH. The pooled adverse event rate for tacrolimus was 25.5% in our study. Overall, neurotoxicity and gastrointestinal issues are the most common side-effects, while diabetes mellitus, nephrotoxicity, pruritus and alopecia may also occur[48]. Previous studies showed that the high serum tacrolimus levels may have led to the rise in creatinine level and subsequent nephrotoxicity. The latest studies suggest using a lower dose of tacrolimus to maintain

blood levels below 6 ng/dL, to prevent probable renal complications or significant changes in creatinine level[32]. Monitoring the drug level to maintain a satisfactory dose and prevent nephrotoxicity is a crucial aspect. Thus, physicians must remain cautious when prescribing tacrolimus at a high dosage. Other reasons for withdrawing tacrolimus treatment include hemolytic uremic syndrome, development of squamous cell carcinoma, intense abdominal pain, non-compliance, overlap with primary sclerosing cholangitis and/or primary biliary cirrhosis, and orthotopic liver transplantation. To date, tacrolimus seems to be a reasonable alternative drug for non-responders, but there is no uniform guideline to explain the dosing schedule and an acceptable safety profile, nor an established monitoring protocol for AIH.

MMF: The use of MMF for AIH is safe in most patients except during pregnancy. It is, however, associated with a large number of side-effects, which vary from mild, to tolerable to toxicity severe enough to necessitate discontinuation of treatment[6]. We found a 24.1% pooled adverse event rate for MMF. Previous meta-analysis estimated a much lower pooled adverse event rate at 14%[16]. Similar to our findings, the most common side-effects of MMF in AIH patients include leukopenia, which can be relieved by reducing the dose, and gastrointestinal issues, in the form of nausea, vomiting and diarrhea[34,37,40-43]. Other, less commonly reported side-effects include severe neutropenia, sepsis, myalgia, pancreatitis, headache, hair loss, and sore gums/sensitive teeth, as well as facial and upper extremity paresthesia[38,40-43]. MMF has major disadvantages in that it is more expensive than AZA and, most importantly, it is teratogenic, which is a major concern in female patients of reproductive age (since AIH affects mainly young females). MMF is contra-indicated during pregnancy, as the United State Food and Drug Administration (FDA) labels it as pregnancy category D. The safety of MMF during pregnancy was not addressed by any of the studies in our meta-analysis.

A note of caution should be added on the retrospective nature of the included studies, which may over- or underestimate the safety profiles of tacrolimus and MMF. Given the long follow-up periods reported and the observed data, both agents appear to be relatively safe alternatives for treating refractory AIH.

Mortality

Despite a good response to first-line treatment, the long-term mortality rate of AIH patients is greater than that of the general population. Untreated AIH can lead to a mortality rate as high as 40% within 6 mo[8,49]. We found a pooled mortality rate of 11.5% for tacrolimus and of 9% for MMF. So far, our study is the first to evaluate the rate for tacrolimus as a second-line therapy in refractory AIH patients. Most of the included studies reported no deaths or only one dead during tacrolimus or MMF therapy. A previous meta-analysis underestimated the pooled mortality rate, which was reported as 7.2% for MMF[15]. Similar to our findings, the largest cohort to date related to second-line treatments in AIH[36] evaluated both tacrolimus and MMF; they reported the highest mortality rate of 11.2% for tacrolimus and 12.4% for MMF. Variation in mortality rates across individual studies may be due to a pre-selection of patients by referral to tertiary centers or to the exclusion of some high-risk patient categories[50,51]. Cohorts showing higher mortality rates have been larger, from multiple centers or from non-tertiary centers, and tended to report on patients who were younger at presentation[36].

Grading of evidence

Rating the quality of evidence by using GRADE is now becoming a recommended step in evidence-based synthesis and it is the most widely adopted tool for grading the quality of evidence and for making recommendations[52]. Previous meta-analyses that addressed the efficacy of tacrolimus and MMF did not include effect estimates for biochemical remission[15] or only provided inconclusive analysis of the effect of MMF as a second-line treatment[16]. Thus, they could not make comprehensive or reliable recommendations. Here we have assessed the evidence using GRADE, which proved to be of very low quality for our primary and secondary outcomes due to the risk of bias and inconsistency. Our analysis therefore offers a starting point for understanding the comprehensive evidence for using tacrolimus and MMF in refractory AIH patients, and our results can provide information for decision-makers, with the ultimate goal of improving clinical outcomes and enhancing patient care.

The natural course of AIH has been clearly outlined and the efficacy of first-line treatment has been well established in naive AIH, although little was known about second-line treatments in refractory AIH. Collectively, our results, along with earlier results, suggest that tacrolimus may be superior to MMF as a therapy in patients not

responding to first-line therapy, while MMF is considered a suitable second-line therapy for patients who are intolerant to the standard treatment. However, these results have been based on observational studies. So far, there have been no clinical trials comparing these treatments directly. In the absence of randomized controlled trials, the existing data on the efficacy of tacrolimus and MMF as second-line treatment modalities for AIH patients remain inconclusive; the current evidence has been mainly derived from several retrospective, mostly single-center, case series or reports[17,18,29-47], or based on expert opinion. This means the evidence is of poor quality due to significant levels of bias. Thus, the reproducibility of results may vary considerably across studies. As a consequence, the recently published guidelines[8,49], have led to great differences in the management of refractory AIH patients. The AASLD guideline states that patients with treatment failure should be managed with higher dose first-line therapy before considering second-line treatments[8]. Whereas the European Association for the Study of the Liver guideline suggests using high dose first-line treatment or alternative medications such as MMF, 6-mercaptopurine or 6-tioguanine, despite reconfirmation of diagnosis and adherence[49]. These differences do not provide any insight into which second-line therapy to consider first for these patients, and emphasize the need to conduct standardized, prospective, and preferably randomized studies, with standardized definitions of therapeutic endpoints[53].

In summary, we found most of the evidence was of low-quality. Given the reported side-effects and mortality rates, we do not feel able to offer recommendations for future research on second-line treatment modalities for refractory AIH. More larger, controlled and prospective studies are required to compare alternative drugs with first-line treatments in patients with AIH.

Study limitations

Our study was limited by the low methodological quality and small numbers of patients in the studies covered by our meta-analysis.

CONCLUSION

Tacrolimus might be a promising alternative drug in the efficient treatment of AIH patients who did not respond to first-line treatments (it was effective in 59.1% of those non-responders treated as second-line), while MMF is considered effective in 73.5% of patients who proved to be intolerant to first-line therapy. However, we found that the quality of evidence is not high, and it is thus questionable whether these results should be worked into a clinical guideline. Well-planned, prospective, multicenter studies of second-line treatments for patients with AIH would help to define the optimal dose, treatment schedule, required duration, and treatment endpoints. In addition, such studies should perform close monitoring of the side-effects. Future prospects in AIH treatments, especially in refractory patients, will be establishing individualized approaches to develop more effective and better tolerated novel therapies.

ARTICLE HIGHLIGHTS

Research background

The standard treatment of autoimmune hepatitis (AIH) is based on corticosteroids, either given alone or in combination with azathioprine, which both lead to remission in 80% of patients. Second-line treatments are needed for patients who have refractory disease. Tacrolimus and mycophenolate mofetil (MMF) have empirically been used the most, as second line treatments for AIH. However, high-quality data on the alternative management of AIH are scarce.

Research motivation

The accumulating but still sparse data indicate that refractory AIH patients do respond to these second-line treatments. However, there is no firm evidence of their effectiveness.

Research objectives

The aims of this study were to evaluate the efficacy and safety of tacrolimus and MMF and the quality of evidence by using the Grading of Recommendations Assessment,

Development and Evaluation approach (GRADE).

Research methods

A systematic review and meta-analysis of the available data were performed. We reviewed the literature, focusing on our aim to identify, appraise, select and synthesize all the high-quality evidence available. We calculated pooled event rates for three outcome measures, defined as biochemical remission, adverse events, and mortality, with their corresponding 95% confidence intervals. Random effects model was applied whenever there was significant heterogeneity between studies. The GRADE approach was used to assess the quality of evidence for primary and secondary outcomes.

Research results

Overall, 21 observational studies, comprising 584 patients with AIH who were unable to tolerate or respond to first-line treatment, met our eligibility criteria. Tacrolimus is efficient in treating patients who did not respond to first-line treatments, yielding a biochemical remission rate of 59.1%, while MMF is considered effective for patients who are intolerant to the first-line therapy, yielding a biochemical remission rate of 73.5%. Moreover, the overall quality assessments using GRADE proved to be very low for all our outcomes in both treatment groups.

Research conclusions

The available evidence shows tacrolimus and MMF are in practice considered effective for AIH patients who are non-responder or intolerant to first-line treatment, but we found no high-quality evidence to support this statement and the translation of these findings to AIH clinical guidelines is questionable.

Research perspectives

Well-planned, prospective, multicenter studies of second-line treatments for patients with AIH would help to define the optimal dose, treatment schedule, required duration, and treatment endpoints. In addition, such studies should perform close monitoring of the side-effects.

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