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Contents

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OPINION REVIEW

Stricter national standards are required for credentialing of endoscopic-retrograde-cholangiopan-creatography in the United States

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Abstract

Endoscopic-retrograde-cholangiopancreatography (ERCP) is now a vital modality with primarily therapeutic and occasionally solely diagnostic utility for numerous biliary/pancreatic disorders. It has a significantly steeper learning curve than that for other standard gastrointestinal (GI) endoscopies, such as esophagogastroduodenoscopy or colonoscopy, due to greater technical difficulty and higher risk of complications. Yet, GI fellows have limited exposure to ERCP during standard-three-year-GI-fellowships because ERCP is much less frequently performed than esophagogastroduodenoscopy/colonoscopy. This led to adding an optional year of training in therapeutic endoscopy. Yet many graduates from standard three-year-fellowships without advanced training intensely pursue independent/unsupervised ERCP privileges despite inadequate numbers of performed ERCPs and unacceptably low rates of successful selective cannulation of desired (biliary or pancreatic) duct. Hospital credentialing committees have traditionally performed ERCP credentialing, but this practice has led to widespread flouting of recommended guidelines (*e.g.*, planned privileging of applicant with 20% successful cannulation rate, or after performing only 7 ERCPs); and intense politicking of committee members by applicants, their practice groups, and potential competitors. Consequently, some gastroenterologists upon completing standard fellowships train and learn ERCP "on the job" during independent/unsupervised practice, which can result in bad outcomes: high rates of failed bile duct cannulation. This severe clinical problem is indicated by publication of ≥ 12 ERCP competency studies/guidelines during

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last 5 years. However, lack of mandatory, quantitative, ERCP credentialing criteria has permitted neglect of recommended guidelines. This work comprehensively reviews literature on ERCP credentialing; reviews rationales for proposed guidelines; reports problems with current system; and proposes novel criteria for competency. This work advocates for mandatory, national, written, minimum, quantitative, standards, including cognitive skills (possibly assessed by a nationwide examination), and technical skills, assessed by number performed (≥ 200-250 ERCPs), types of ERCPs, success rate (approximately ≥ 90% cannulation of desired duct), and letters of recommendation by program director/ERCP mentor. Mandatory criteria should ideally not be monitored by a hospital committee subjected to intense politicking by applicants, their employers, and sometimes even competitors, but an independent national entity, like the National Board of Medical Examiners/American Board of Internal Medicine.

Key words: Endoscopic retrograde cholangiopancreatography; Privileges; Credentialing; Gastroenterology fellowship training; Advanced gastrointestinal endoscopy training; Certification; Standards

Core tip: An additional, optional year of endoscopic-retrograde-cholangiopancreatography (ERCP) training was added because of limited ERCP exposure during standard-three-year-gastrointestinal-fellowships and its greater endoscopic technical difficulty. Yet, many graduates from standard-three-year-fellowships intensely pursue ERCP privileges despite inadequate numbers of ERCPs, or low successful duct cannulation rates. Hospital credentialing committees have sometimes disregarded recommended ERCP credentialing guidelines. Consequently, some gastroenterologists learn ERCP "on the job", after completing standard GI fellowships, during unsupervised practice. National, *mandatory*, standards for ERCP are advocated, including number (≥ 200-250) of performed ERCPs, and ≥ 85%-90% successful cannulation rate. An independent entity should oversee ERCP credentialing to prevent politicking within hospital committees.

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INTRODUCTION

Endoscopic retrograde cholangiopancreatography (ERCP) compared to the other standard gastrointestinal (GI) procedures of esophagogastroduodenoscopy (EGD) and colonoscopy is technically far more difficult, requires greater skill, and entails a higher rate of clinically significant complications. Yet GI fellows have limited exposure to ERCP during a standard three years GI fellowship because ERCP is much less frequently performed than EGD or colonoscopy. This combination of a steep learning curve and limited exposure motivated the institution of an optional extra year of advanced endoscopy training, primarily devoted to ERCP but also devoted to endoscopic ultrasound (EUS). Yet many graduates from standard three years fellowships without an extra year of advanced training intensely pursue independent (unsupervised) ERCP privileges[1]. Sometimes these requests are reasonable, but they may be unjustified if the applicant did not perform an adequate number of ERCPs during standard fellowship training and has a low rate of successful selective cannulation of the desired (biliary or pancreatic) duct[1-3]. At least 18 studies, recommended guidelines, and editorials on ERCP credentialing have been published in the last 30 years, including 12 published since 2015 (Table 1). However, lack of mandatory, quantitative, written, criteria for ERCP credentialing has permitted ambiguities and neglect of recommended guidelines. Consequently, some

gastroenterologists upon completing standard fellowship training learn ERCP "on the job" during independent unsupervised practice, which can result in bad ERCP outcomes: Extremely high rates of failed bile duct cannulation which necessitates that patients undergo repeat ERCP by another gastroenterologist at another time[2].

Fifty years after the institution of ERCP in 1968, this opinion piece calls for establishment of mandatory, written, and quantitative national criteria to prevent ambiguities and disregard of recommended guidelines. The monitor of the mandatory criteria should not ideally be an in-hospital committee because this committee is subject to intense political pressure by the applicants themselves and their employers (personal unpublished data, Cappell as Chief of Gastroenterology and Hepatology for last 12 years), but a truly independent entity. This work suggests consideration of establishing an independent national board, similar to the National Board of Medical Examiners or American Board of Internal Medicine, to maintain uniform national standards divorced from political pressure by local applicants, their private practice groups, competing gastroenterology groups, or hospitals. This work reviews prior recommended criteria for ERCP credentialing and their rationale; the compelling need for quantitative, mandatory criteria; and provides an example of mandatory credentialing criteria, which are merely illustrative because criteria should be established by consensus of a committee of ERCP experts preferably assembled under the auspices of the American Society of Gastrointestinal Endoscopy (ASGE), or similar professional GI organization.

METHODS

Literature on ERCP credentialing and training was comprehensively searched by computer using PubMed and Ovid with the following medical subject headings/keywords: ("ERCP" OR "endoscopic retrograde cholangiopancreatography" OR "endoscopic retrograde cholangiography") AND ("privileges" OR "privileging" OR "credentials" OR "credentialing" OR "guidelines" OR "position paper" OR "recommendation" OR "American Society for Gastrointestinal Endoscopy" OR "ASGE" OR "competence" OR "competency" OR "competent" OR "training" OR "trainee" OR "quality" OR "independent practice"). The two authors independently performed literature searches, and decided on which articles to incorporate into this review according to appropriateness of article content and article priority based on consensus. This review was rendered up-to-date by repeating a computerized literature search just before submitting this work for publication which identified one new article just e-published ahead of print one week before submission of this opinion piece[4].

This work is restricted to privileging of adult gastroenterologists for ERCP in the United States because practice patterns, standards of care, and medical malpractice litigation patterns differ in the rest of the world; and excludes ERCP credentialing for pediatric gastroenterologists, or GI, hepatobiliary, and pancreatic surgeons because they have different practice patterns.

RESULTS

History and clinical significance of ERCP

ERCP is currently the procedure of choice for many biliary and pancreatic disorders. About 350000-500000 ERCP's are currently performed annually in the United States[3,5]. Common indications include choledocholithiasis, obstructive jaundice, biliary pancreatitis, malignant biliary obstruction, and benign biliary strictures; while uncommon indications include recurrent pancreatitis of unknown etiology, biliary or pancreatic duct leaks, pancreatic stones, pancreatic strictures, chronic pancreatitis, and sphincter of Oddi dysfunction[6,7].

After William McCune, an obstetrician, performed the first ERCP in 1968, Peter Cotton, a gastroenterologist, reported a clinical series of 60 diagnostic ERCPs in 1972[8]. Critical developments in diagnostic ERCP technology included side-viewing endoscopes to view the mural papilla en face, cannulation catheters, endoscopic elevators to facilitate papillary cannulation, guide-wires, biopsy forceps, and brushes. Classen and Demling in Germany[9], and Kawai and colleagues in Japan[10], pioneered ERCP therapy using sphincterotomes to open the ampulla and endoscopic devices to extract choledocholithiasis. ERCP has become increasingly therapeutic because of critical advances in therapeutic technology, including sphincterotomes for sphincterotomy, inflatable balloons or stents to dilate strictures, electrocautery to stem hemobilia, and baskets or inflatable balloons to retrieve choledocholithiasis.

Table 1 Literature review of criteria for endoscopic retrograde cholangiopancreatography privileging and practice

First author, Journal, Year study published	Type of study	Proposed Minimum number of ERCPs or other criteria for determining competence	Quality indicator or comments
Wigton *et al*[37], American College of Physicians, Ann Intern Med 1988	Position paper, American College of Physicians	35 supervised ERCPs. No quality indicators specified.	Document to include degree of success of ERCP. Types of ERCP not specified.
Watkins *et al*[38], Gastrointest Endosc 1996	Original prospective report of point at which GI fellow achieves 85% rate of cannulation of both pancreatic and bile ducts	100 supervised ERCPs	Point at which GI fellow achieves 85% rate of cannulation of desired duct (either pancreatic duct or bile duct)
Jowell *et al*[40], Ann Intern Med 1996	Prospective study involved grading of 1796 ERCPs among 17 GI fellows	180 supervised ERCPs	Number of ERCPs for individual skills: 160 for cholangiography, 160 for pancreatic duct cannulation, 120 for stone extraction, and 60 for stent insertion.
Eisen *et al*[41], Gastrointest Endosc 2002	Position paper, American Society for Gastrointestinal Endoscopy	180 supervised ERCPs	80% ability to cannulate the duct of interest (either bile duct or pancreatic duct)
Garcia-Cano[39], Surg Endosc 2007	Letter to editor based on personal experience as surgeon training in ERCP	200 ERCPs	Based on personal experience at point at which achieved 80% rate of cannulation of bile duct. Anecdotal evidence.
Verma *et al*[43], Gastrointest Endosc 2007	Retrospective review of single operator ERCP learning curve	> 80% rate of successful deep cannulation of bile duct	Achieved at performing 350-400 ERCPs
Shahidi *et al*[42], Gastrointest Endosc 2015	Systematic review encompassing 9 studies	Competency achieved after 79 to 300 ERCPs, depending upon learning curve of individual trainee	Competency for specific quality indicators: 70 to 160 ERCPs for pancreatic duct cannulation, and 160 to 400 ERCPs for deep bile duct cannulation
Cotton[26], Gastrointest Endosc 2015	Editorial	Supports guidelines set by Australian Conjoint Committee of 200 ERCPs	To include a minimum of 80 sphincterotomies with intact papillary sphincters, and a minimum of 60 stents
Adler *et al*[44], Gastrointest Endosc 2015	Position paper, American Society for Gastrointestinal Endoscopy Quality Assurance in Endoscopy Committee	> 90% rate of deep cannulation of duct of interest with native papilla, > 90% rate of extraction of common bile duct stone < 1 cm in patient with normal bile duct anatomy, > 90% successful stent placement in patient with normal anatomy	---
Ekkelenkamp *et al*[47], Endoscopy 2015[47]	Nationwide analysis of 8575 ERCPs by 171 endoscopists in Holland during 1 yr.	Overall rate of "successful" ERCPs was 83.4% for native papilla and 89.4% after sphincterotomy.	Provides a reasonable estimate of expected success rate for ERCP operators.
Wani *et al*[48], Gastrointest Endosc 2016	Prospective multicenter trial conducted among 5 advanced GI endoscopy fellows at 5 medical centers	Number of ERCPs to achieve successful cannulation rate > 90% of biliary duct varied from 26 to 211 ERCPs.	Demonstrates variability in learning curves to achieve competence in ERCP as determined by > 90% rate of bile duct cannulation
Wani *et al*[49], Clin Gastroenterol Hepatol 2017	Prospective multicenter study of 22 advanced GI endoscopy trainees at 20 medical centers	Demonstrated substantial variability in learning curves for cognitive and technical ability in ERCP. This suggests basing criteria for competence not on volume, but on achieving landmarks for quality indicators (*e.g.*, successful cannulation rate).	Variable learning curves for achieving cognitive and technical success in ERCPs upon completion of advanced endoscopy fellowship
Wani *et al*[45], Gastrointest Endosc 2018	Gastrointestinal Endoscopy white paper	Developed comprehensive data collection and reporting tool for assessing ERCP performance	Demonstrated feasibility of using a central database to monitor GI fellow performance
Faulx *et al*[50], Gastrointest Endosc 2017	American Society for Gastrointestinal Endoscopy Standards of Practice Committee Guideline	200 supervised ERCP procedures for assessing competency. Additionally, independently perform > 80 sphincterotomies and > 60 biliary stent placements	
Wani *et al*[53], Gastroenterology 2018	Prospective multicenter clinical trial involving 22 advanced GI endoscopy fellows	After completing an advanced endoscopy fellowship, ERCP operators achieved an average successful cannulation rate of 94.9% in private practice.	This work shows that advanced endoscopy fellowship training leads to successful ERCP performance in private practice.

Cotton[60], Gastrointest Endoscopy 2017	Survey of 1126 responding United States gastroenterologists	No written guidelines for initial ERCP credentialing-21%. No written guidelines for repeat credentialing process-54%.	Urgent need to improve credentialing process.
Cassani *et al*[63], Gastrointest Endosc 2017	Editorial on sorry state of ERCP credentialing	"Despite repeated studies, editorials, gastroenterology society papers, credentialing committees have yet to take the initiative and require increased scrutiny for both hospital and (ERCP) procedural outcomes."	Frank discussion of current failures in credentialing process.
Wani *et al*[4], Gastrointest Endoscopy 2019	Prospective multicenter study determining standards for competency for ERCP based on learning curves for 37 advanced endoscopy fellows in 32 programs.	Advanced endoscopy fellow required an average of 226 ERCPs to achieve competency in native papillary cannulation, and required an average of 120 sphincterotomies to achieve competency in biliary sphincterotomy.	Provides guidance on threshold number of ERCPs at which to assess competency.

ERCP: Endoscopic retrograde cholangiopancreatography; GI: Gastrointestinal.

Therapeutic ERCP often produces dramatic cures of life-threatening conditions, and is less invasive and safer than surgical options for various disorders, such as ascending cholangitis from choledocholithiasis[11]. ERCP therapies have largely obviated surgery for choledocholithiasis and choledochal strictures, and can improve survival in patients with cholangiocarcinoma[12,13]. The relatively recent change of ERCP to become a predominantly therapeutic modality has also been fostered by development of less invasive and safer pancreatico-biliary diagnostic tests than ERCP, including EUS and magnetic resonance cholangiopancreatography (MRCP).

ERCP entails greater risks of clinically significant complications than other standard GI endoscopic procedures, such as EGD or colonoscopy[14]. ERCP has a reported mortality ranging from 0.2%[15] to 1%[16], depending upon patient age, medical status, and planned therapeutic intervention. Kalaitzakis *et al*[17] reported a dramatic 12% mortality at 3 mo from post-ERCP pancreatitis, though patient-related factors, including cancer diagnosis and advanced age were contributing factors. Moreover, ultimately fatal post-ERCP pancreatitis is probably under-reported[18-20]. Life-threatening complications after ERCP and sphincterotomy include post-sphincterotomy bleeding, unremitting cholangitis, bile leak, and duodenal perforation. ERCP is a relatively common cause of medical malpractice litigation against gastroenterologists, and engenders a much higher rate of medical malpractice suits than other GI endoscopic procedures[21].

ERCP is uncommonly performed relative to the other two standard GI endoscopic procedures of EGD and colonoscopy. For example, at William Beaumont Hospital in Royal Oak, one of the ten largest hospitals in the United States, ERCP represents only about 700 (3%) of a total of 24000 annual GI endoscopies. Moreover, this percentage probably overestimates its relative frequency in the United States because this tertiary hospital is a referral center for ERCPs. The technically demanding skills and relatively high risks of severe complications of ERCP vs. relatively infrequent exposure to ERCP during standard three years of GI fellowship prompted gastroenterologist-administrators to establish a year of advanced endoscopy fellowship training primarily devoted to ERCP. Advanced endoscopy programs also generally incorporate training in diagnostic and therapeutic EUS, and increasingly offer other relatively recently introduced advanced endoscopic procedures, including: Double balloon enteroscopy, ablation therapy for Barrett's mucosa, endoscopic mucosal resection, endoscopic submucosal dissection, peroral endoscopic myotomoy, endoscopic clips to close GI perforations, endoscopic suturing, and peroral cholangiopancreatoscopy. Advanced GI fellows also need to generate clinical income by performing routine EGDs and colonoscopies because their salaries are not funded by Medicare. Training in these other advanced techniques and performance of routine GI endoscopies can adulterate the advanced fellowship experience in ERCP. The core curriculum outlines ERCP trainee goals in terms of expectations and experiences[22,23] (Table 2). In one survey, graduating advanced fellows were generally satisfied with their advanced endoscopy training, but some of them would have skipped the extra year of advanced training altogether if they had more exposure to ERCP and EUS during their standard GI fellowship[24].

History and rationale of ERCP credentialing criteria

After the introduction of diagnostic ERCP in 1968 and therapeutic ERCP in 1974, clinical demand for ERCP burgeoned with scant regulation of ERCP privileges

Table 2 Core Curriculum for endoscopic-retrograde-cholangiopancreatography trainees

Cognitive
1 Obtain written, witnessed, and informed patient consent with discussion of the indication for the ERCP; potential complications including pancreatitis, hemorrhage, duct leak, perforation and infection; alternative tests or therapies; and adequately answer patient questions
2 Realize appropriate indications for ERCP and accessory interventions
3 Evaluate patient prior to procedure and optimize outcomes, in terms of potential bleeding (*i.e.*, hold antiplatelet and anticoagulants if possible), and administer antibiotics as necessary to prevent subsequent sepsis
4 Understand and practice prophylactic interventions, especially to prevent post-ERCP pancreatitis
5 Know "best practice" recommendations as to technical approaches during ERCP
6 Knowledge of optimal management of ERCP complications
7 Manage the patient after ERCP as in-patient or outpatient, as appropriate
8 Manage complications occurring during or after ERCP
9 Knowledgeably discuss findings and consult with allied specialists: hepatobiliary or pancreatic surgeons and interventional radiologists
Technical (not comprehensive)
1 Evaluate ampulla in a knowledgeable fashion
2 Access necessary ductal system via deep cannulation ≥ 90% attempts
3 Procure required fluoroscopic images of the biliary and pancreatic ductal systems
4 Working knowledge to interpret fluoroscopic images
5 Perform optimal biliary and/or pancreatic sphincterotomy as required
6 Extract biliary and pancreatic duct stones via basket or balloon.
7 Insert plastic and metal stents into pancreatic and biliary system as required
8 Perform intraductal endoscopy and associated diagnostic or therapeutic maneuvers, as required: EHL, laser, biopsies, and brushings

Adopted with major modifications from Jorgensen *et al*[23]. ERCP: Endoscopic retrograde cholangiopancreatography; EHL: electrohydraulic lithotripsy.

because of a severe shortage of endoscopists trained and proficient in this novel procedure. Cappell vividly recalls how Jerry Siegel, a highly talented pioneer clinical ERCP practitioner, travelled to numerous major academic hospitals throughout New York City totting a briefcase containing his own ERCP endoscope and endoscopic accessories to perform ERCP on referred patients, after being granted temporary, emergency, ERCP privileges at these hospitals. This lightly regulated "Wild West" of "have ERCP scope will travel" was prevalent in the mid-to-late 1970s[25]. Notable other ERCP pioneers included Peter Cotton at Duke University Hospital who has become the primary advocate of tighter regulation of ERCP privileges, Meinhard Classen in Germany, and Ito in Japan.

Now 50 years after its inauguration, mandatory, written, and strict regulations of training and credentialing of ERCP, based on national guidelines, should be adopted. Regulation is required because of: (1) High risks inherent to ERCP, especially of post-ERCP pancreatitis, bile leaks, and post-sphincterotomy bleeding; (2) Extremely high level of technical expertise and cognitive skills needed to master therapeutic ERCP, especially given the ever increasingly innovative and more sophisticated therapeutic technologies; and (3) Diverse ERCP training backgrounds of applicants for ERCP privileges. As GI Division Director, Dr. Cappell and the Credentials Committee members have "denied" five applicants' applications for ERCP privileges because of insufficient documentation of ERCP training during their standard three year GI fellowship, low rate of bile duct cannulation, or request to perform ERCP at this tertiary university hospital while primarily based at a satellite hospital without arranging for emergency coverage to handle post ERCP emergencies on their patients at the academic teaching hospital. Indeed, one GI attending was denied ERCP privileges at a major academic hospital because of an extremely low volume of ERCP during a standard three year GI fellowship, and only a 20% rate of biliary cannulation afterwards in clinical practice[2]. Cotton[26] reported in 2015 that one GI fellow upon entering private GI practice was requested to join the ERCP rotation after having performed precisely 7 ERCPs during a standard three year GI fellowship. Contrariwise, applicants may be granted ERCP privileges despite inadequate training during a standard three years GI fellowship due to political pressure from a prominent GI group that the applicant is joining (Cappell, personal unpublished data). Such credentialing problems are exceedingly rare for other endoscopic procedures (Cappell, personal experience as Chief of Gastroenterology and Hepatology at an academic medical center during the last 12 years).

Concerns regarding ERCP training, competency, standard of practice, and

credentialing began to appear in the literature about 25 years after its introduction[27,28]. At the time, few medical institutions offered one year advanced endoscopy training programs focused on diagnostic and therapeutic ERCP after standard GI fellowship. Graduating GI fellows who did not train via this extra year were still eligible to obtain ERCP privileges if they had adequate training and experience during the standard three years of GI fellowship, pursued extra training abroad, or simply were self-trained. The landscape began to change around the new millennium as advanced training programs became relatively common. About 66 advanced endoscopy training programs currently exist in the United States. Concurrently, administrators of advanced endoscopy fellowships formulated a core curriculum to help standardize ERCP training and practice[22,29]. The ASGE has resolved that competency in ERCP is not required during standard GI fellowship training, and strongly recommended advanced endoscopy fellowship training to achieve clinical competence in ERCP[22,30]. Despite these recommendations, a survey of GI fellows graduating from standard three year GI fellowships reported in 2003 that 91% of them intended to perform ERCP, even though only one-third had met ERCP volume thresholds for independent ERCP practice during fellowship training[1]. This disparity between clinical practice and professional guidelines continues to some extent even today, despite criticism of "low-volume" operators and endorsement of quality metrics, including highly successful biliary cannulation rates and low ERCP complication rates[3,31,32]. For example, a survey conducted in 2015 showed that 40% of graduating third year GI fellows believed that they would be able to perform ERCP independently upon graduation, even though only 19% of them had performed ≥ 200 ERCPs[26].

Competency

ERCP competency is defined as thoroughly understanding the cognitive aspects of ERCP, and reliably achieving its technical goals[33]. Patient outcome, patient satisfaction, and ERCP complication profile are also important. Criteria for competency, however, still remain controversial during the present era of numerous advanced GI fellowships (Table 3). One perspective suggested that biliary cannulation rate is a better parameter to assess competency than absolute ERCP numbers[34]. ERCP practitioners and trainees should incorporate best practice guidelines and prevention strategies to minimize complications, especially to prevent post-ERCP pancreatitis[35,36]. Overestimating ERCP skills or embellishing credentials can have dire clinical consequences[2].

Competency in ERCP was initially determined by numbers performed during GI fellowship training. An early study recommended an extremely low threshold of 35 ERCPs to achieve competency[37]. A prospective six-year study suggested a minimal threshold of 100 ERCPs to achieve competency, with a > 85% biliary cannulation rate[38]. An important prospective study set 180-200 ERCPs as the threshold for competency, including 120 gallstone extractions, and 60 stent deployments[39]. An anecdotal Spanish study similarly recommended 200 ERCPs as the threshold for competency for surgeons[40], as was also recommended for gastroenterologists by the ASGE Standards of Practice Committee[41]. A systematic review, published in 2015, reported that in five studies, the minimum threshold for competency, as determined by very high rate of successful selective, duct cannulation, ranged from 79 to 300 ERCPs[42]. Cotton[26] published an editorial applauding these findings, and reiterating that too many low-volume operators were performing ERCP.

Recent published recommendations have become stricter. The Mayo Clinic study[43] recommended > 350 ERCPs performed on a native papilla as a threshold for competency. Other studies included ERCPs on patients with prior sphincterotomy that renders biliary cannulation much easier. Recent studies suggest a ≥ 90% selective cannulation rate is an appropriate metric for a native ampulla[44], but a "competent" ERCP operator should attain a ≥ 95% rate in a papilla status post sphincterotomy or with precut maneuvers[43].

Assessment of ERCP competency based solely on numbers is flawed because of wide variability in: training programs, individual experiences within given training programs, exposure to ERCP during standard three-year GI fellowships, innate endoscopic ability of individual trainees, and difficulty in translating training results during GI fellowship into clinical practice[45]. For example, individual GI fellow experience at a given ERCP may vary from passive observation, attempting only one bile duct cannulation, performing the diagnostic ERCP, or performing the entire diagnostic and therapeutic ERCP. Also, individual learning curves for ERCP are non-linear. Relative difficulty of ERCP procedures can be graded according to patient characteristics (*e.g.*, stable patient *vs* acutely septic patient from ascending cholangitis), biliary anatomy (*e.g.*, status post Billroth II *vs* native anatomy), procedural indication (*e.g.*, obstructive jaundice *vs* recurrent idiopathic pancreatitis), and procedure intervention (*e.g.*, solely diagnostic *vs* sphincterotomy and stone

Table 3 Ongoing controversies in endoscopic-retrograde-cholangiopancreatography training and privileging

Ongoing controversies in endoscopic-retrograde-cholangiopancreatography training and privileging
1 What minimum number (if any) of ERCPs should be performed during a dedicated advanced training fellowship to justify credentialing?
2 What minimum number of ERCPs should be documented by a physician who seeks credentialing in ERCP after completing a standard 3 yr GI fellowship?
3 What should the profile of submitted ERCPs consist of in terms of therapeutic interventions?
4 Should all new physicians granted ERCP privileges have a probation period with monitoring by a proctor, and if so for how long?
5 What criteria, other than numbers, should be used to assess competency in ERCP?
Cannulation of desired duct(s)
Procedure outcome
Patient outcome
Complication rate
Monitoring of ERCPs during a probation period.
6 Should administrators of standard gastroenterology fellowship training programs that are 3 yr long be allowed to certify GI fellows in ERCP or should credentialing be restricted only to GI fellows who have completed an extra year of advanced endoscopy training?
7 Is post-training proctoring acceptable as a means to attain ERCP competency?
8 Should EUS training be mandatory for ERCP performance?
9 Should advanced GI fellowship training programs offer only a dedicated EUS or ERCP pathway but not both?
10 Should curriculum content for advanced therapeutic training be nationally standardized? Are there a sufficient number of advanced GI endoscopy program fellowships and are they of sufficient duration?
11 Should manpower concerns affect ERCP credentialing or should standards for competency be the only consideration?
12 Should individual hospital needs for ERCP operators affect credentialing?
13 Should all ERCP practitioners be compelled to participate in an on-call rotation for emergency ERCPs to be performed at night or on weekends?
14 Should all GI endoscopists with staff privileges for ERCP be compelled to join a rotation to perform ERCPs on public uninsured patients?
15 Should all ERCP practitioners be required to perform a minimum number of ERCPs per annum to maintain ERCP privileges (proficiency)? If so, what is the minimum number: 25 or 50 ERCPs per annum?
16 Should a national board exam, similar in concept to the examination in Gastroenterology by the American Board of Medicine be required for certification in ERCP to assess cognitive knowledge in ERCP and related clinical disciplines?

ERCP: Endoscopic retrograde cholangiopancreatography; GI: Gastrointestinal.

extraction for acute cholangitis). A reported grading system is useful to compare data on individual trainees or from different studies on ERCP competency[46] (Table 4).

A recent trend is to emphasize learning curves rather than mere numbers. A Dutch study showed widely variable individual rates of acquisition of cognitive ability and technical skills, with a steeper learning curve for selective cannulation than for other technical skills, such as stent deployment[47]. A sophisticated multi-center American study of > 1000 advanced endoscopy trainees reported a wide range of individual acquisition of cognitive ability and technical skills, which was only mildly-to-moderately correlated with ERCP volume[48]. Another large American study found that only 60% of advanced endoscopy trainees attained technical ERCP competence, even though all trainees achieved cognitive competency[49]. This study demonstrated the feasibility of a central database to determine individual learning curves for ERCP. The authors called their database tool TEESAT, an acronym for The EUS and ERCP Skills Assessment Tool. The 2017 ASGE Practice Guidelines[50] recommended that ≥ 200 ERCP's should be performed before competency is assessed, and that this minimum threshold should include > 80 sphincterotomies, and > 60 biliary stent deployments. A national board examination may be required in the future to assess cognitive ERCP skills.

Training

Advanced endoscopy fellowship training will eventually become the predominant route for ERCP practice. Paradigm shifts regarding advanced endoscopy training during the last two decades include: (1) Not mandating ERCP training during standard GI fellowships; (2) Exponential increase in number of advanced endoscopic fellowship training programs[51]; and (3) Recent transition from an "apprenticeship" to "milestones" model for medical education[30,31,52]. In the apprenticeship model, trainees are evaluated in relation to their peers at the same year of fellowship training. In the milestone model, trainees are evaluated by reaching appropriate interim milestones until they are evaluated for the ultimate milestone at graduation of competence for "independent practice". The ASGE permits trainees to designate preference for

Table 4 Grading System for endoscopic-retrograde-cholangiopancreatography difficulty

Grading system for ERCP
Grade I
Deep cannulation of CBD or main pancreatic duct
Extraction of small-to-medium (≤ 10 mm) biliary stones
Biliary stenting for leaks
Grade II
Treatment of extra-hepatic benign or malignant ductal strictures
Placement of prophylactic pancreatic stents
Extraction of larger biliary stones
Grade III
Pancreatic stricture dilation and stenting
Removal of mobile pancreatic stones ≤ 5 mm
Hilar tumor stenting
Treatment of hilar and intrahepatic biliary stricture
Sphincter of Oddi manometry
Limited pancreatic sphincterotomy
Removal of migrated pancreatic stents
Grade IV
Removal of impacted and larger pancreatic stones
Pseudocyst drainage or necrosecetomy
Ampullectomy
ERCP in patient with altered anatomy (*e.g.*, status post Billroth II surgery)
Minor papilla therapy
Grade V
"Rendezvous" procedure to access and stent the biliary and pancreatic systems - requires endoscopic ultrasound training

Adapted with modifications from Cotton[3]. ERCP: Endoscopic retrograde cholangiopancreatography; CBD: Common bile duct.

training in EUS, ERCP or both, but applicants are generally more interested in ERCP, which is a more highly valued and marketable skill.

Under the milestones paradigm, most advanced GI fellows achieved cognitive and technical goals, and were judged ready for independent practice[53], but the rate of skill acquisition was highly variable[42,45,48,49]. Number of ERCPs is deemed relevant only as a threshold to initiate formal assessment of achieving milestones for ERCP skills[45].

Various endoscopic simulators, including mechanical devices, virtual (computer-generated) models, organ explants, and live animals can help teach and train GI fellows in ERCP[51]. An inexpensive, simple, fabricated device boosted trainee confidence in performing actual ERCPs[54], while an elaborate, expensive European mechanical simulator was also helpful[55]. Computer simulators can improve ERCP skills[56]. Trainees can practice cannulation, sphincterotomy, and stent deployment using neo-papillae and neo-bile duct fashioned from chicken heart and trachea, respectively[57]. Simulators are useful adjuncts to formal training, but cannot replace actual clinical experience. Experienced ERCP operators can further improve their hands-on skills with workshops, such as those offered by the ASGE, especially for training in new and emerging technologies[58,59].

Credentialing

Credentialing is potentially contentious. A gastroenterologist without ERCP privileges is barred from performing ERCP. One-time denial is potentially tantamount to lifelong denial of this privilege because ERCP skills generally atrophy over time with disuse. Denial may decrease professional reputation because ERCP is perceived as a prestigious endoscopic procedure, and may decrease referrals for standard GI endoscopies because the gastroenterologist may be perceived as incapable of performing complex endoscopies.

Credential committee members have legitimate concerns about patient safety and potential malpractice litigation if applicants with borderline credentials are granted ERCP privileges, but the committee members could theoretically be biased against granting ERCP privileges to newly graduating GI attendings to stifle competition. However, institutional manpower needs and economic incentives may trump such

concerns because patients with pancreatico-biliary diseases could be rerouted or transferred by ambulance to other hospitals if a given hospital has too few GI attendings with ERCP privileges.

A recent survey demonstrated that 21% of United States hospitals lack formal guidelines for initial credentialing for ERCP privileges, 59% of them lack formal guidelines for renewal of such privileges, 67% of them do not collect data on sphincterotomy rate or volume, and 85% of them do not collect data on rates of successful biliary cannulation[60]. After performing this survey, Cotton *et al*[60] reiterated his plea for adherence to credentialing guidelines, and establishing standardized national certification for ERCP. He recommended different criteria for initial credentialing for ERCP after completing GI fellowship, credentialing after one year of GI practice, and subsequent credentialing for renewal of ERCP privileges. Clinical studies suggest that 40 to 50 ERCP with sphincterotomies annually is a reasonable number to maintain ERCP proficiency, as evidenced by such high volume operators having a lower risk of ERCP complications than low volume operators[61,62]. The accompanying editorial endorsed Cotton's proposal, called the current credentialing process "alarming", and urged credential committees to analyze more data on ERCP outcomes and hospital course[63]. Publications on ERCP competency including original articles, position papers, recommended guidelines, and editorials are listed in Table 5. Most authorities believe that endoscopists performing high volumes of ERCPs generally provide higher quality ERCPs and improve patient outcome compared to endoscopists performing low volumes of ERCPs[31,64-66]. Low-volume operators derive less personal satisfaction from performing ERCP, possibly because of greater stress, and may be viewed less favorably by endoscopy personnel[65].

GI endoscopists who perform ERCPs at several hospitals pose another problem. How can endoscopists who rarely perform ERCPs at a given hospital be evaluated for re-credentialing based on the limited data available at this given hospital? Who manages patient complications after ERCP when the performing gastroenterologist is away at another hospital? Should all endoscopists with ERCP privileges be compelled to participate in on-call rotations for emergency ERCPs that must be performed at night or on weekends, and should all of them be compelled to participate in a rotation to perform ERCPs on patients without medical insurance? At Beaumont Hospital at Royal Oak, renewal of privileges has been linked to enrolling in an on-call rotation for emergency ERCPs and in a rotation for uninsured patients requiring ERCPs.

Manpower needs

Few studies analyze United States manpower needs for ERCP. The approximately 350000-500000 ERCPs performed annually in the United States[3,5] are mostly performed by endoscopists without advanced endoscopy training, and this predominance will likely persist for years to come. In Cotton's survey published in 2017[60,63], only one-quarter of surveyed ERCP operators in the United States had advanced ERCP training, and these practitioners typically practiced in academic urban or suburban hospitals. Rigorous vetting of applicants for ERCP privileges could limit the number of operators. Rigorous vetting should work well in densely populated urban areas with high concentrations of ERCP operators, but may be problematic in rural and inner-city hospitals that are likely underserved in number of ERCP operators. This phenomenon may explain the reluctance of some hospitals to rigorously follow professional ERCP guidelines. Transferring patients from inner city or rural hospitals to academic medical centers for emergency ERCPs, for indications such as acute cholangitis or bile leaks, is problematic. Gastroenterologists at low-volume ERCP centers may solicit medical advice by telephone or video communications from ERCP experts at high-volume centers[67]. To adapt to local shortages of gastroenterologists performing ERCPs, surgeons could increase their rate of performing intraoperative cholangiography and could potentially perform ERCP themselves[68,69], while interventional radiologists could perform transhepatic cholangiography as a substitute for ERCP.

The duties of the individual GI fellow applying for ERCP privileges, of the supervisory attending, and of the credentialing committee at which the GI fellow is applying for ERCP privileges upon completion of the fellowship are summarized in Table 5. Upon graduation of a GI fellow, training programs should issue a nationally standardized certificate regarding ERCPs that provides quantitative data on numbers of ERCPs and percentages of successful therapeutic interventions (proposed ERCP report card illustrated in Table 6). Credentialing should grant preference to trainees who performed an extra year of GI fellowship mostly devoted to ERCP training.

This work has proposed that national criteria be mandatory rather than recommended. One reasonable method of enforcement is for chairs of credentialing committees to certify that the physician was granted ERCP privileges in accord with

Table 5 Determining competency for endoscopic-retrograde-cholangiopancreatography

Duties of GI fellow applying for ERCP privileges at a hospital	Duties of GI supervisor of ERCP training/GI fellowship program director	Duties of hospital committee voting on ERCP privileges for applying physician
Contemporaneously sign each ERCP note in which actively participated	Ascertain GI fellow signs ERCP notes when the fellow actively participated in case	
Can contemporaneously sign each note in which merely observed ERCP, but observer status should be reflected in note	Allow fellow to sign on note as an observer (not active participant) in cases in which fellow was passive observer	Passive observation of an ERCP should be meaningful in enhancing cognitive skills for ERCP, but cannot count towards minimum threshold for performed ERCPs
ERCP note in which GI fellow participated should specify what technical procedures performed during ERCP: *e.g.*, sphincterotomy, stone extraction, dilation of stenosis, and *etc.*	Ascertain that endoscopy report includes all technical aspects of the performed ERCP	Ascertain that received data is complete
Collate all numbers performed for ERCP: Number performed, number (%) cannulated, number with sphincterotomy, number with stone retrieval, number of strictures dilated, and *etc.*	Review total numbers of ERCPs and number of ERCPs in which special techniques were employed as appropriate. Record rate (%) of success of special techniques.	Review total numbers of ERCPs and number (%) of ERCP special procedures and determine whether these data satisfy minimal numbers and minimal % of successful result required for competency
Make sure packet with ERCP numbers and recommendations is submitted in a timely manner at hospital applicant is applying for ERCP privileges	Sign form containing total number of ERCPs, and write whether GI fellow is recommended for independent privileges in ERCP	Decide in a timely manner whether physician granted ERCP privileges. If decision is negative, provide internal due process to appeal decision

Duties of graduating gastrointestinal fellow, endoscopic-retrograde-cholangiopancreatography supervisors during fellowship training, and credentialing committee at hospital to which applicant is applying for endoscopic-retrograde-cholangiopancreatography privileges. ERCP: Endoscopic retrograde cholangiopancreatography; GI: Gastrointestinal.

the national criteria. This certification may, however, prove to be an inadequate remedy. An ultimate solution is to establish a Board for ERCP certification similar to the American Board of Internal Medicine that would remove politically difficult decisions on privileging from hospital committees. Cappell has personal experiences of enduring political pressures during 6 cases of applicants denied ERCP privileges and in 1 case of an applicant approved ERCP privileges despite borderline credentials (personal unpublished data, Cappell). Requiring certification by a national board would dissociate deliberations from local political considerations and would avoid flouting of the numerous recommended guidelines, position papers, and recommendations promulgated during the past 30 years.

MRCP and EUS

The advent of MRCP and EUS has improved the landscape so that ERCP is now rarely indicated solely for diagnosis. Current diagnostic ERCP indications are restricted to subtle primary sclerosing cholangitis, chronic pancreatitis, and indeterminate biliary strictures[70]. MRCP is limited by contraindications from implanted metal devices, high imaging cost, technical expertise required for performance and interpretation, and occasionally claustrophobia. EUS is often performed by the same operator who would perform the contemplated ERCP. Acquisition of endoscopic skills in both ERCP and EUS is therefore highly desirable during advanced GI fellowship training. A "negative" MRCP or EUS can obviate the need for ERCP[71] in about 70% and 50% of cases, respectively[72]. Such avoidance of ERCP is desirable because patients without evident malignancy or choledocholithiasis may be more susceptible to ERCP-induced pancreatitis[73].

CONCLUSION

ERCP training and credentialing has become a growing concern during the last thirty years. ERCP differs from most other endoscopic procedures in its predominantly therapeutic intent, necessity for typical performance in hospitals, steep learning curve, and penchant for occasionally causing severe complications. ERCP training has undergone several paradigms shifts during the past 50 years including: (1) Change to not requiring ERCP training during standard GI fellowship; (2) Recommendation for training in advanced endoscopy fellowships to obtain privileges in ERCP; (3) Recent exponential growth in number of advanced endoscopy fellowships; and (4) recent shift in ERCP training from an apprenticeship to milestone model, which emphasizes progressive milestones in competence until ultimately achieving independent practice at graduation. Advanced fellows and advanced fellowships have been increasingly

Table 6 Proposed standardized gastroenterology fellowship report card for endoscopic-retrograde-cholangiopancreatography training and performance

Proposed standardized gastroenterology fellowship report card for ERCP
Achievements:
_____ Number of ERCPs in which trainee was only a passive observer.
_____ Number of ERCPs in which trainee actively participated (excludes ERCPs in which trainee was only a passive observer)
_____ Number and _____% of ERCPs in which trainee personally successfully cannulated at least one duct (includes either common bile duct or pancreatic duct)
_____ Number and _____% of ERCPs in which trainee personally successfully performed sphincterotomy
_____ Number and _____% of ERCPs in which trainee successfully personally performed stone retrieval by basket or balloon pull through
_____ Number of ERCPs in which trainee successfully dilated a biliary or pancreatic stricture
_____ Number of ERCPs in which trainee successfully personally deployed a stent
_____ Number of ERCPs in which trainee successfully retrieved a stent
_____ Number of ERCPs in which trainee successfully used daughter endoscope (*e.g.*, Spyscope) technology
Adverse events:
Number and percent of total ERCPs in which trainee participated in which adverse events occurred:
For fatal complication: Number_____ Percent of total _____
For major adverse events: Number _____ Percent of total _____
For minor adverse events: Number _____ Percent of total _____
Has the trainee been a defendant in a medical malpractice suit in any ERCP in which the trainee participated? _____ Yes _____ No.
Has the trainee had privileges in ERCP revoked or restricted or received a written warning? _____Yes _____No
Has the trainee voluntarily given up ERCP privileges in lieu of these privileges being revoked or restricted? ___Yes _____No

ERCP: Endoscopic retrograde cholangiopancreatography.

scrutinized regarding ERCP skills, as reflected by at least 12 publications on this subject during the past 5 years (Table 1), including analysis of trainee learning curves and criteria for ultimate competency. ERCP authorities frequently call to improve standardization of ERCP competence and performance, including quality metrics, such as high rates of successful biliary cannulation and low rates of procedural complications. The advanced endoscopy-trained pool remains relatively limited, and most ERCP operators have been trained during a standard GI fellowship or by other means. Hospital credentialing committees have to balance patient safety and risk of medical malpractice litigation versus real-world needs for available ERCP operators and desire for increased hospital revenue from treating patients requiring ERCPs. Credentialing in ERCP by any route other than advanced endoscopy training is expected to become increasingly difficult.

All hospitals need to establish or adopt written criteria for ERCP privileges that are standardized according to national guidelines. Hospitals should be granted a transition period to implement these criteria. Criteria should include minimum number of ERCPs required to apply for privileges and minimum annual volume to maintain privileges. These criteria may specify numbers required for specialized ERCP procedures, including sphincterotomy, stricture dilatation, stent deployment, stone extractions, and per-oral cholangiopancreatoscopy. Hospitals will have to develop criteria for minimally acceptable rates of successful biliary cannulation, sphincterotomy, and gallstone extraction. Hospitals may also have to determine maximal acceptable rates of major post-ERCP complications, especially for ERCP-induced pancreatitis, clinically significant post-sphincterotomy bleeding, and bile leaks. These criteria would benefit hospitals by establishing firm criteria for granting versus denying ERCP privileges, protect applying physicians from being denied ERCP privileges for competitive rather than professional reasons, and protect patients from inadequately trained ERCP operators. Institutional GI morbidity and mortality committees should review all mortality from major ERCP complications including ERCP-induced pancreatitis, post-sphincterotomy bleeding, bile leaks, or duodenal perforations.

Implementing and following stricter ERCP regulations would likely introduce new direct costs from the work required to closely monitor ERCP practitioners, and indirect costs from reduction in the number of ERCP operators. Public health administrators need to realize that increased regulation is costly and budget these inherent costs to benefit patient care. Criteria for ERCP competency still remain controversial in 2019 and are sometimes flouted by hospitals despite the numerous

studies, position papers, editorials, and recommended guidelines for ERCP competency. Mandatory criteria monitored by a national board, similar to the National Board of Medical Examiners or American Board of Internal Medicine, would provide nationally uniform criteria, which would be divorced from local political considerations of individual practitioners, their GI groups, competing GI groups, and given hospital, and would avoid widespread flouting of recommended guidelines.

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OPINION REVIEW

Colorectal peritoneal metastases: Optimal management review

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Abstract

The peritoneum is a common site of dissemination for colorrectal cancer, with a poorer prognosis than other sites of metastases. In the last two decades, it has been considered as a locoregional disease progression and treated as such with curative intention treatments. Cytoreductive surgery (CRS) and hyperthermic intraperitoneal chemotherapy (HIPEC) is the actual reference treatment for these patients as better survival results have been reached as compared to systemic chemotherapy alone, but its therapeutic efficacy is still under debate. Actual guidelines recommend that the management of colorectal cancer with peritoneal metastases should be led by a multidisciplinary team carried out in experienced centers and consider CRS + HIPEC for selected patients. Accumulative evidence in the last three years suggests that this is a curative treatment that may improve patients disease-free survival, decrease the risk of recurrence, and does not increase the risk of treatment-related mortality. In this review we aim to gather the latest results from referral centers and opinions from experts about the effectiveness and feasibility of CRS + HIPEC for treating peritoneal disease from colorectal malignancies.

Key words: Peritoneal metastases; Colorectal cancer; Cytoreductive surgery; Hyperthermic intraperitoneal chemotherapy; Peritoneal carcinomatosis

Core tip: Patients with peritoneal metastases from colorectal cancer have classically been associated with limited survival and treated only with palliative surgery and systemic support. Cytoreductive surgery and hyperthermic intraperitoneal chemotherapy, often combined with systemic treatment, are increasingly performed with a curative intent for well-selected patients. Recent data suggests an important improvement of overall and disease-free survival for these patients. This article aims to review the state of art for the management of peritoneal metastases from colorectal origin and to confine the latest experts' consensus and future directives.

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INTRODUCTION

We conducted a literature review to provide a comprehensive and updated overview of the actual management of colorectal cancer (CRC) with peritoneal metastases (PM) as the only site of spread. Our specific purpose is to enhance our understanding of the following aspects of this disease: (1) To know the biological pathway for PM, the concept of its locoregional spread, and the associated genetic and molecular factors; (2) To update our knowledge about the prognosis factors of peritoneal disease; (3) To delve into the multidisciplinary approach of peritoneal metastatic CRC, both synchronous and metachronous; (4) To show the recent scientific evidence on clinical trials and meta-analyses on disease-free and overall survival after treatment with cytoreductive surgery (CRS) plus hyperthermic intraperitoneal chemotherapy (HIPEC); and (5) To show the new and more promising investigation lines and trials for the future management of peritoneal metastatic CRC.

EPIDEMIOLOGY

CRC is the third most common cancer and the second most common cancer-related mortality globally. Patients have a favorable prognosis when diagnosed at an early stage: 70%-80% are eligible for curative-intent surgery, with a 5-year survival of 72%-93% for stages I-II[1]. Approximately 25% of the remaining patients present metastases at the time of diagnosis[2]. Among these individuals, up to 8% have synchronous peritoneal carcinomatosis, and approximately 20% already have liver metastases[3]. Recurrent or systemic disease during the follow-up period after curative treatment of the primary tumor will develop in 20%-30% of patients. Half of these recurrences will develop liver metastases[3].

Although it was believed that metachronous PM occur in less than 10% of cases of CRC, being the third most frequent site of recurrence after liver and lung, its prevalence is still not well known. As an example of the underestimation, due to the lack of reliability of traditional imaging and unspecific symptomatology, one study of autopsied patients that did from CRC reported an incidence of 40%-80% unknown metachronous peritoneal carcinomatosis[4]. The peritoneum is the only dissemination location in 4.8% of cases and is more frequent for colon tumors (5.7%) than for rectal tumors (1.7%)[2]. Thus, thorough studies must be performed to exclude another site of metastatic disease because of the high possibility of further spread.

PATHOPHYSIOLOGY OF COLORECTAL PERITONEAL METASTASES

Peritoneal seeding as a dissemination pathway of an invasive cancer is believed to be the final result of the specific expression of oncogenes and binding proteins that allow the detachment of tumor cells to proliferate in the peritoneal environment[5]. The actual deep knowledge of genetics and molecular cancer mechanisms is becoming a strong tool to determine the likelihood for peritoneal spread, to avoid locoregional relapse after the first curative surgery and to assess the real implication on prognosis.

The molecular genetic influence

Many different genetic alterations characterize the two main pathways that have been described for the development of CRC: The conventional adenoma–carcinoma pathway and the serrated pathway. Notably, 80% of sporadic cases of CRC involve chromosomal instability, which includes the molecular targets for most of the novel chemotherapy agents, including K-RAS, B-RAF or pT53. Another group encompasses microsatellite instability (MSI) as a result of inactivation, mutation and/or epigenetic alteration of mismatch repair genes. This mechanism is one of the main causes of hereditary nonpolyposis CRC but also entails 10%-20% of sporadic CRCs. Depending

on the proportion and type of microsatellite marker mutation, these tumors are classified into two groups: High (MSI-H) and low MSI or microsatellite stability (MSI-L/MSS)[2,6,7].

MSI-H has been reported as a better prognosis condition, with a lower metastatic potential for distant recurrence than MSI-L tumors. However, a large single-center study[8] on outcomes for CRCs with MSI showed that MSI-H recurrences, most of them located on the peritoneum, had a worse survival than MSI-L carcinomas. These studies showed that most of these relapses are not eligible for curative resection and that this type of tumor progression is able to avoid immune mechanisms of protection by several means, which increases its malignant potential. Advanced tumor stages for MSI-H tumors have also been associated with BRAF mutations[9].

The BRAF V600E mutation is observed in 10% of CRCs, and it has been widely related to a worse prognosis. A recent meta-analysis described a more than two times higher risk of mortality in patients carrying this alteration[10]. The mild response to modern chemotherapy is attributed to a frequent acquired resistance to BRAF-inhibitor development[11]. This mutation has also been strongly associated with PM[12]. However, other studies have reported a more encouraging prognosis when the BRAF V600E mutation is found in early-stage cancers[13] or when the mutation is a non-V600E BRAF mutation[14].

KRAS mutations are present in up to 40% of CRCs sporadic cases, and evidence has shown that codon 12 KRAS mutations encompass a negative prognosis but not codon 13 KRAS mutations[15]. This biomarker has also been reported as a risk factor for a worse prognosis in patients with PM from CRC origin, and its detection has played a major role in patient selection for CRC+HIPEC in recent years[16]. A new oncogenic mechanism that constrains the tumor cell phenotype switch, called the epithelial-mesenchymal transition, has been suggested to be an aggressive subtype for the high rates of this mutation detected on carcinomatous nodules in a recent publication[17].

Peritoneal seeding theory

Peritoneal implants are believed to be the consequences of primary abdominal tumor cell detachment or malignant cell dissemination during surgical manipulation of the tumor when the margins of resection are very close and for lymphatic or blood vessel transection. These cells attach to the peritoneum as a result of the molecular interaction between cancer cells and host elements, and invade the subperitoneal layer, where angiogenesis promotes their growth. Furthermore, numerous metachronous peritoneal implants developed along the surgical planes that were opened during the first surgery, becoming trapped by fibrin as part of the healing process, which can be a difficult location to reach by systemic chemotherapy[5].

CLINICAL PRESENTATION

Risk factors for developing peritoneal metastases from colorectal cancer

Population-based studies agree on a set of risk factors for developing metachronous PM for CRC, which includes the stage at diagnosis (incidence for pT4 stage, established as an independent risk factor, has been reported to be 17%-50%; and for pT3 stage of 5%-10%)[18,19], intraabdominal colon location, principally right-side colon cancer, infiltrative and ulcero-infiltrative carcinomas, mucinous adenocarcinoma, younger patients than 70-75 years, emergency procedures because of obstructive or perforated cancer at diagnosis, lymph node metastases and nonradical oncological resection during the first surgery[20-22] (Table 1). By knowing which patients are more likely to develop peritoneal spread of the disease, several prophylactic or early detection strategies have been designed, as listed below.

Complicated disease presentation: Recommendation for management

Clinical presentation of intestinal obstruction and/or perforation involves a poor prognosis, independent of stage. Even uncommon, if this situation occurs in the context of a synchronous PM scenario, with the main aim of offering the best prognosis possible to these patients, the expert global recommendations include the following: (1) To perform the minimal surgical action needed to resolve the emergency situation. Primary resection should only be performed in perforated tumors. Obstructed patients should be treated by creating derivative stomas. Non-obstructed tumors should not be resected but treated by stomas or stents, although colonic stenting should be avoided in patient candidates for antiangiogenic agents because of higher rates of perforation reported[1]; (2) Always provide adequate biopsies of the primary tumor and/or peritoneal implants; (3) Describe the extension of the peritoneal disease using the peritoneal cancer index (PCI) score. If limited peritoneal

Table 1 Risk factors for metachronous peritoneal metastases

Risk factors for metachronous peritoneal metastases
Advanced T stage
Lymph node metastases
Synchronous ovarian metastases
Poor differentiation
Colon origin (versus rectal origin)
Uncomplete primary tumor resection
Mucinous adenocarcinoma
Signet ring histology
Emergency surgery at diagnosis
Young age

disease is found, there is still a high recommendation to not perform surgical resection in the emergency context because it does not add a better free-disease survival and could hinder a better combined treatment modality[23,24].

THE NATURAL EVOLUTION OF COLORECTAL PERITONEAL DISEASE

PM is a negative prognostic factor in patients with metastatic CRC. Patients with isolated nonperitoneal sites (including liver and lungs) had significantly better overall survival than that of patients with isolated peritoneal metastatic CRC. A recent large cohort study[25] showed that the combination of peritoneal involvement with two nonperitoneal sites had a similar survival compared with peritoneal metastasis alone. In fact, given the poor prognosis of PM itself, in the eighth edition of the tumor-node-metastasis classification published in 2017, CRC with peritoneal metastasis is categorized as M1c, with or without other organ involvement, separately from M1a (one organ metastases) and M1b (≥ two organ metastases).

Historically, as peritoneal carcinomatosis was considered a terminal stage of disease, patients used to receive only supportive treatment or palliative chemotherapy. Generally, survival did not reach 6 mo, and patients were extremely symptomatic because of abdominal distension, intestinal obstruction and tumoral cachexia for constitutional syndrome[26]. Palliative surgery was not a better option, since it reached a high perioperative mortality and morbidity (over 12% and 22%, respectively)[27]. Currently, the best survival reported for only systemic modern chemotherapy and supportive care for PM from colorectal origin is 15.2-23.4 mo [28,29]. These poor results prompted the need to find a more effective approach for this stage of disease.

CYTOREDUCTIVE SURGERY AND HYPERTHERMIC INTRAPERITONEAL CHEMOTHERAPY

Why should this treatment be considered?

In the 1980s, according to Sugarbaker *et al*[30] publications, peritoneal carcinomatosis ceased to be considered a systemic metastatic disease and therefore a terminal condition. Currently, peritoneal carcinomatosis is referred to as PM, and it is determined to be a locoregional spread that is eligible for a curative intent approach based on optimal CRS plus HIPEC[25,28,31-34]. This treatment entails a major, expensive and complex surgery that requires an optimal selection of the patients, with an adequate performance status and an accurate preoperative extension study, and the key for the best survival outcomes is to ensure a complete cytoreduction with no residual tumor remaining. Therefore, a presurgical study is paramount to optimizing the indications of the patients who would benefit the most from this treatment.

Patients selection

The performance status of the patient is a fundamental aspect considering the morbidity of CRC+HIPEC. Eastern Cooperative Oncology Group or World Health Organization indices > 2 and serious comorbidities (severe cardiopulmonary or renal failure) are considered major contraindications[35]. Age is a factor to be considered

globally, but there is no cut-off to contraindicate CRS plus HIPEC.

Preoperative scores: Tools for prognosis and surgical indication

Peritoneal carcinomatosis index: PCI is the most accepted score for both evaluating the tumor burden and estimating the patient prognosis, suggesting this score as a helpful tool for surgical indication as well (Figure 1). Faron *et al*[36] showed a significant relationship between two factors: 5-year survival was noticeably higher (53%) for PCI < 10, up to 23% for PCI between 10-20 and only 12% for PCI > 20. Currently, the majority of guidelines accept a PCI > 20 as a contraindication for CRS plus HIPEC[37].

Peritoneal surface disease severity score: The peritoneal surface disease severity score (PSDSS) is another commonly used and validated preoperative severity score. Some experts consider a PSDSS > IV (10 points) a contraindication for CRS due to its ominous outcomes. No additional benefit has been shown for PSDSS over PCI[38].

Completeness of the cytoreduction score: Completeness of the cytoreduction score is another useful tool widely used to assess prognosis. A complete resection of macroscopic tumors is a necessary requirement for the long-term benefit of CRS; thus, incomplete resections or debulking have shown survival improvement[32,39]. In other words, CRS must not be performed if less than a CC1 (< 2.5 mm) cytoreduction cannot be assured (for peritoneal disease, that small amount of residual tumor is expected to be eradicated by HIPEC)[40].

Resectability

For every patient diagnosed with CRC, abdomen and chest computed tomography (CT) and complete colonoscopy (or a CT colonography if the former cannot be done[41]) with biopsies for histopathological study must be performed. Positron emission tomography (PET) is not routinely recommended by the experts, although PET is considered a helpful tool for a more truthful evaluation of the tumor extension in cases of extra-abdominal disease suspicion and for obtaining additional information on equivocal lesions. Special attention should be paid to the detection of radiological signs of peritoneal metastasis on the abdominal CT scan to contemplate the best treatment approach for the patient. These signs include ascites, mesenteric effacement, peritoneal nodules or masses, luminar narrowing, peritoneal thickening and enhancement (Figure 2).

Assessment of a preoperative PCI with imaging would allow a preliminary evaluation of complete tumor resectability that can be useful to avoid unnecessary surgeries. However, a nonnegligible rate of inaccuracy between radiological PCI and surgical PCI has been observed, mainly because of the underestimation of tumor burden and operator dependence[42]. Implant sizes less than 5 mm and locations are the main factors for missing disease[43]. Magnetic resonance imaging has been reported to have a higher sensitivity than CT scan, especially for implants located on the small bowel (quadrants 9 to 12) and for unexperienced radiologists[42,44]. Combining both techniques can also increase the precision of preoperatory estimation[45].

Sugarbaker *et al*[46] proposed a series of radiologic features that could predict, if two or more are present, unresectability, suboptimal surgical resection or complex resections due to the tumor burden. PET/CT has demonstrated good sensitivity and specificity for the detection of peritoneal disease[47]. However, its accuracy can be altered in cases of small implants, mucinous and gastric tumors, as well as under some inflammatory conditions (*e.g.*, inflammatory bowel disease or abscesses).

Consideration of synchronous lymph node infiltration

Lymph node infiltration is widely recognized as a poor prognosis factor for recurrence in the setting of the primary tumor. Publications on this theme also note that lymph node metastases present a more aggressive tumor biology at the time of CRS+HIPEC and have a dismal effect on survival[48,49].

Consideration of synchronous liver metastases

Concurrent liver and PM were initially considered a nonresectable condition due to the poor prognosis. However, the more recent consideration of liver implants with a poorer metastatic potential than peritoneal lesions and its excellent response (up to 60%) to modern systemic chemotherapy have changed the minds of surgeons concerning its approach[a].

In the last decade, several publications have suggested the feasibility and shown the survival improvement of liver metastases resection without adding morbidity[50,51]. There is no consensus on the number of liver metastases that limits the indication for CRS plus HIPEC as long as complete resection can be fulfilled[52]. Elias developed a nomogram to estimate the prognosis of patients according to the number of liver metastases and the PCI[53].

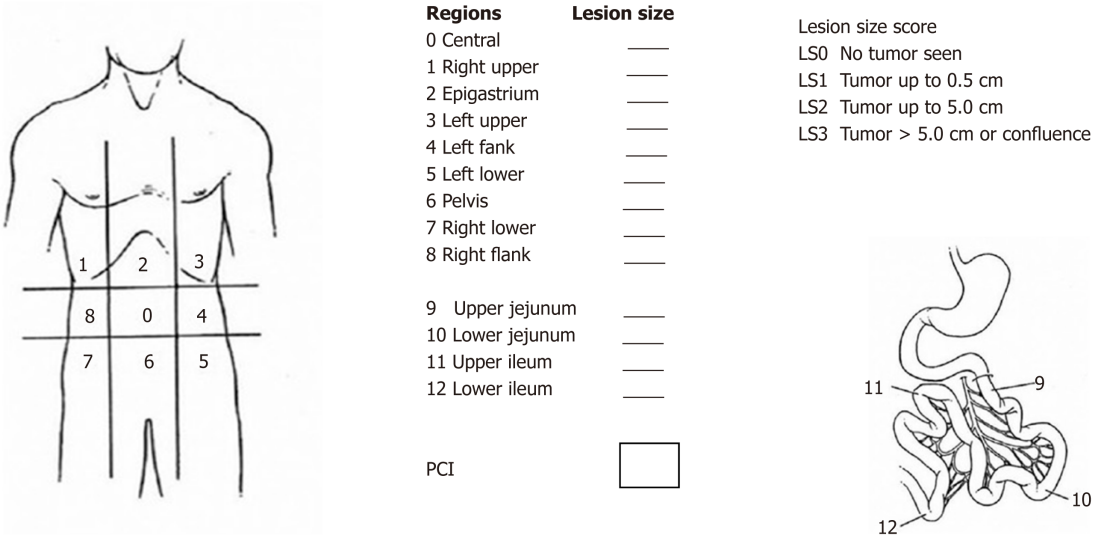

Figure 1 Peritoneal carcinomatosis index described by Sugarbaker P.

Although the presence and amount of liver disease are no longer a contraindication for CRS plus HIPEC, a recent meta-analysis on the survival benefit of these patients found a negative or no significant impact on survival in most of the studies compared to patients with PM alone[54]. However, ablation or resection of liver metastases has proven to offer better survival compared to palliative treatment[55]. Therefore, multidisciplinary consensus and individual evaluation must be considered for every particular case.

Absolute exclusion criteria for CRS plus HIPEC

Absolute exclusion criteria for CRS plus HIPEC: Bulky and/or diffuse peritoneal metastasis (Figure 3); Unresectable extra-abdominal metastases; Vast small bowel serosa or small bowel mesentery involvement; Multi-segmentary malignant bowel obstruction or non-affected length of small bowel < 150 cm; Massive affectation of the hepatic hilus; The presence of unresectable liver metastases or the requirement of a major hepatectomy, which could lead to insufficient hepatic function.

THE ROLE OF SYSTEMIC CHEMOTHERAPY

The effectiveness of neoadjuvant and adjuvant systemic chemotherapy for patients with CRS and PM has long been controversial among different publications.

Neoadjuvant chemotherapy

With the present experience, no survival benefits can be attributed to the administration of neoadjuvant chemotherapy for PM from CRC origin, without extra-abdominal disease. A recent systematic review found no strong evidence for its efficacy regarding overall survival[56]. Neoadjuvancy has only shown survival improvement in univariate analysis in some publications, and even in two papers[57,58] multivariate analysis suggested a worse median survival when neoadjuvant chemotherapy was used. A prospective study reported the first experience using modern systemic chemotherapy with and without biological agents[59] and showed no effects on unresectable disease, considering a minimal study sample and a high percentage of unfavorable histology.

Notably, there are wide and non-standardized chemotherapy regimens used for the different teams and no randomized controlled trial has been performed in this context, which hinders the potential implication of this therapy. Similarly, there is no reliable data concerning the safety of the surgery following neoadjuvant chemotherapy with biological agents, such as bevacizumab, a vascular endothelial growth factor inhibitor that has been suggested as a risk factor for anastomotic leaks due to its implication in tissue regeneration. There are few publications on this issue, initially brought to light by Eveno *et al*[60], whose retrospective analysis showed a statistically significant increase in major morbidity (mainly because of intraabdominal abscesses) when bevacizumab was included in the neoadjuvant treatment. Subsequently, Ceelen *et al*[61] published their experience using bevacizumab neoadjuvant regimens, and they

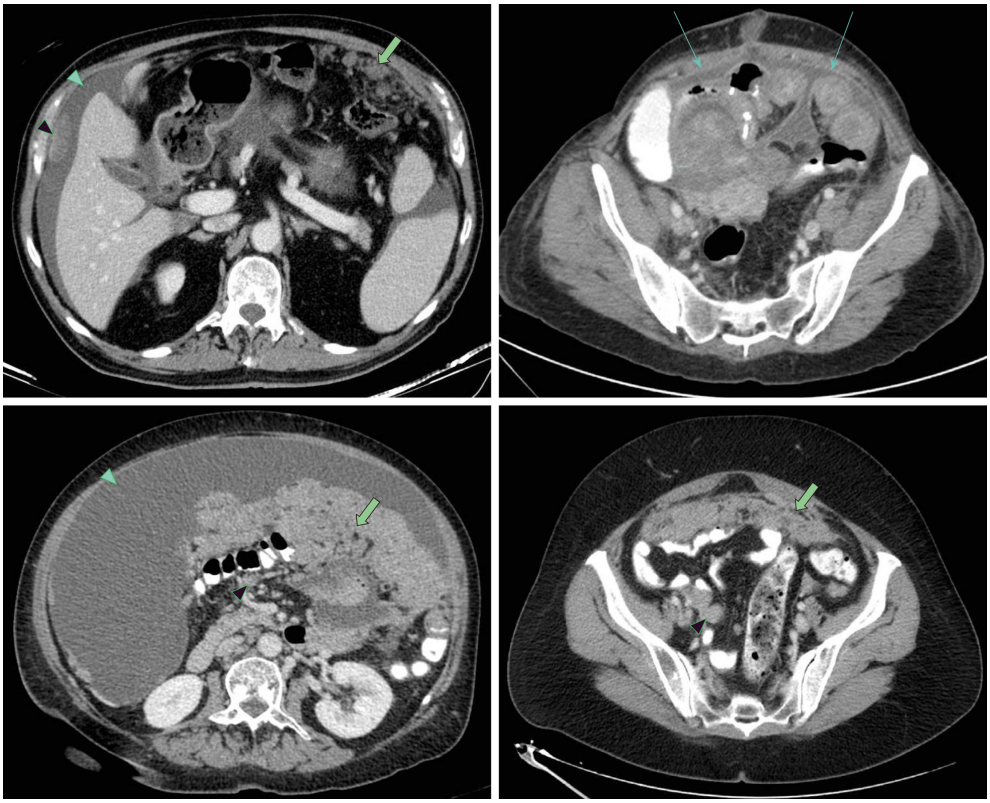

Figure 2 Radiological computed tomography signs for peritoneal disease. Wide green arrow: Omental cake; Thin green arrow: Peritoneal thickening; Green arrow-head: Malignant ascites; Black arrow-head: Peritoneal nodules.

not only found worsening of the postoperative morbidity but also reported a beneficial effect on overall survival. Other recent publications describe no major postoperative complications related to the use of bevacizumab in the neoadjuvant protocol[62,63], but there are no further studies specifically on CRC.

Adjuvant chemotherapy

The same systematic review[56] suggests the positive effect of adjuvant chemotherapy on overall survival, despite the heterogeneity of the studies. The most recent publications concur on reporting improvement in median survival with modern versus standard chemotherapy protocols[64], and these authors also agree on the actual approach of M1c as a curable stage for CRC[65]. The clinical value of biological therapies remains uncertain. The concept of blocking angiogenesis was a new promising tool for metastatic CRC and has gained popularity in recent years. Previous publications described no increase in survival using these agents after resectable or unresectable disease[64,66]. Conversely, a recent meta-analysis[67] on the different types of anti-VEGF antibody combination therapies has shown a significant improvement in progression-free survival, overall survival and response rate.

The number of adjuvant chemotherapy cycles did not demonstrate a clear relationship with survival in previous publications[56]. A recent study[68] encompassing six phase III trials evaluating the noninferiority of 3 versus 6 months administration of adjuvant modern chemotherapies with either FOLFOX (fluorouracil, leucovorin, and oxaliplatin) or CAPOX (capecitabine and oxaliplatin) was conducted. Among 12384 patients with stage III colon cancer, the 6-month duration of FOLFOX therapy increased the rate of disease-free survival, particularly among patients with high-risk cancers (T4, N2, or both). However, efficacy was maintained with the 3-month duration for low-risk patients and for the CAPOX regimen, which suggests that this protocol could be evaluated to prevent adverse effects, such as persistent neurotoxicity associated with oxaliplatin.

However, the most important independent factor for a better survival is the radical resection of the tumor with curative intent[69]; thus, the effectiveness of any treatment highly depends on the extent of the tumor and the completeness of cytoreduction, attempting to avoid a delay of adjuvant treatment due to surgical complications.

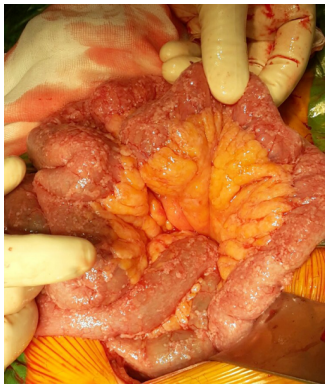

Figure 3 Diffuse miliary carcinomatosis on the small bowel as an example of contraindication for complete cytoreductive surgery.

OUTCOMES OF CYTOREDUCTIVE SURGERY PLUS HYPERTHERMIC INTRAPERITONEAL CHEMOTHERAPY

Morbidity and mortality

A large meta-analysis[65] found that intention-to-cure treatments improved overall survival in patients with CRC + PM better than palliative strategies. The risks reported have progressively decreased (recent studies report 1%-5% mortality rates at centers of excellence). The morbidity associated with curative treatments was higher, but it did not increase the risk of treatment-related mortality or caused an early termination of the treatment. Additionally, the reported morbidity rates are similar to those of other major abdominal procedures: Increased treatment-related complications, longer hospital stays and higher rates of short-term readmission[33,70,71] (Table 2).

A consideration that should be highlighted is that experience and learning curve play an important role in the morbidity and mortality outcomes, so it is strongly recommended that patients should be treated in experienced centers, mentored by specialized institutions for peritoneal diseases, such as PSOGI[70]. Regarding the laparoscopic approach, HIPEC delivery by the laparoscopic approach has already been accepted as a safe and feasible procedure that is performed for different indications. However, there is a lack of knowledge about the oncological quality of laparoscopy CRS for CRC with peritoneal implants. Some groups have published their early experiences in this field; all of these reports suggested the careful selection for thin patients with a PCI < 10 to ensure a complete resection[72-74].

Survival outcomes

In recent years, CRS plus HIPEC plus systemic chemotherapy (both neoadjuvant and after surgery) comprise the multidisciplinary treatment performed in most of the referral centers. Overall survival results with this management can reach up to 62 mo if optimal cytoreduction is achieved (this means, following the cytoreduction completeness score: CCO, no tumor nodules left or CC1, implants ≤ 2.5 mm in maximum dimension)[25]. Increasing evidence from large multicenter cohort studies and some randomized controlled trials suggests that, in selected patients, CRS plus HIPEC as a combined management definitely provides improved overall and disease-free survival compared to that conferred by systemic chemotherapy alone, and possibly a major part of this benefit has to be attributed to cytoreduction[25,28,31,34,65,75] (Table 3).

A recent review of current guidelines about the management of patients with colorectal PM shows no definitive agreement about the role of CRS plus HIPEC, but most of them recommend this approach as a standard therapy for selected patients, reaching a consensus in 71% with a level of evidence 1b[35]. The treatment approach for these patients should always be assessed by a multidisciplinary team discussion, which includes at least a surgeon (ideally, an oncologist surgeon), an oncologist and a radiologist expert[52]. Considering these concepts, the latest National Comprehensive Cancer Network (NCCN) guidelines[32] recommend that CRS plus HIPEC for colorectal PM be considered only at experienced centers for selected patients with limited PM for whom complete cytoreduction is deemed to be achieved.

The role of HIPEC

The lack of consensus about the role of HIPEC may be due to several reasons: The marked heterogeneity of protocols, drugs, carrier solutions and methods of HIPEC

Table 2 Patient and operative factors associated with cytoreductive and hyperthermic intraperitoneal chemotherapy morbidity (modified from Newton *et al*[70])

Patients characteristics	Operative factors
Age > 60-70 yr	Pancreatic resections
Performance status	Bowel resection and anastomosis
Hypoalbuminemia	Surgeon experience
	Peritoneal carcinomatosis index

administration (open, semi-open, closed techniques) and the discrepancy concerning patient eligibility and lack of randomized trials in the era of modern chemotherapy and targeted therapy.

The preliminary results of the PRODIGE 7 trial[76], presented at the American Society of Clinical Oncology (ASCO) meeting in 2018, questioned the widespread conviction of the beneficial effects of HIPEC. After complete cytoreduction of M1c CRC, 265 patients were randomized to standard treatment plus HIPEC with oxaliplatin or standard treatment alone. No significant difference in overall survival was found, with a median of 41.7 months in the HIPEC arm *vs* 41.2 mo in the non-HIPEC arm [Hazard ratio (HR) = 1.00, 95% confidence interval (CI): 0.73-1.37] and no significant difference in relapse-free survival (13.1 *vs* 11.1 mo, HR = 0.90, 95%CI: 0.69-1.90). However, a trend toward better disease-free survival was found on the Kaplan-Meier curves for the first 18 months after surgery, and a subgroup analysis for patients with a PCI between 11 and 15 showed significantly better overall and recurrence-free survival for the HIPEC group.

Regarding morbidity, the study reported a higher late, grade 3-5 morbidity (up to 60 d after surgery) in the HIPEC arm (24.1% *vs* 13.6%, P = 0.03). The unexpected results have encouraged the scientific community to continue searching for the role of HIPEC in PM, as its advantageous effects have been extensively reported in the biomedical literature for CRC and recently proven for other origins[77]. To our knowledge, high quality and complete cytoreduction has been confirmed once again as a pivotal pillar of treatment for peritoneal dissemination of CRC. Efforts are now focused on electing patients who would benefit the most from HIPEC because this trial remarks high PCI as an already known impaired factor.

Another goal is to ascertain the real morbidity (as most of the publications only report the 30-day morbidity-mortality and have widely been compared, similar to other major abdominal surgeries[78]) and reduce the side-effects of HIPEC[79]. This may be achieved by either minimizing drug doses (which has been one critic of the PRODIGE 7, considering previous experimental studies)[80], establishing the benefits of hyperthermia alone and combined with the chemotherapy agents, or trying different drugs or delivery systems. Additionally, the final results are published; to date, only one multicenter randomized trial studying the effects of HIPEC vs standard treatment for patients with established PM of CRC origin (NCT02179489). Therefore, the search is ongoing, and further trials are needed to determine what HIPEC can offer.

REITERATIVE CYTOREDUCTIVE PROCEDURES

Approximately 70-80% of the patients undergoing CRS plus HIPEC will develop recurrence disease, despite the curative intention of this approach. Half of these recurrences will be confined to the peritoneal cavity[81]. This reality has led to the study of the feasibility and safety of reiterative CRS and even HIPEC procedures in recent years. The morbidity and mortality of these surgeries are similar to those of the first procedure in high volume centers[82]. Furthermore, this active approach to recurrent abdominal disease has reported a median survival from 39 to 42.9 mo, a clearly better long-term survival compared to that obtained with systemic treatment alone[82-84]

Keeping in mind that HIPEC therapy has not been proven to be an independent risk factor itself for postoperative complications[71] and that the morbidity reported for CRS in experienced centers is similar to that in other major surgeries, the combined CRS plus HIPEC approach seems an acceptable strategy for this poor stage of disease, as studies continue to show further evidence. Moreover, traditional adjuvant systemic chemotherapy can still be performed following surgery (as it would be the only treatment if surgery could not be performed or it would be rejected). Issues such as the adjunctive contribution of intraperitoneal chemotherapy to CRS and optimal

Table 3 Survival of patients with peritoneal metastases from colorectal cancer treated by cytoreductive surgery plus hyperthermic intraperitoneal chemotherapy

Author/ Years/ Country	1-yr SR (%)	3-yr SR (%)	5-yr SR (%)	Mortality rate (%)	Morbidity rate (%)	Median OS (mo)	OS 95%CI (mo)	PFS 95%CI (mo)	DFS/RFS 95%CI (mo)	Follow-up times (range) (mo)
Controlled studies										
Franko/2010/America[109]	92	51	28	NR	NR	34.7	NR	NR	NR	NR
Gervais/2013/Canada[110]	92	61	36	4	20	54	NR	NR	8	22.8 (2-81)
Goéré/2015/France[111]	90	52	32	5.8	29.5	35	NR	NR	NR	60 (47-74)
Huang/2014/China[112]	63.6	16	NR	0	28.6	13.7	10.0-16.5	NR	NR	41.5 (11.5-70.9)
HIPEC single arm studies										
Cao/2009/Australia[113]	83.6	51.4	32.1	NR	NR	37.0	1-72	NR	NR	19 (1-72)
Ceelen/2014/Belgium[61]	75 (NNT) 75 (NCA) 96 (NCB)	39 (NNT) 30 (NCA) 71 (NCB)	25 (NNT) 13 (NCA)	NR	NR	25 (NNT) 22 (NCA) 39 (NCB) 30 (AC) 22 NAC)	19.1-30.9 (NNT) 12.9-31.1 (NCA) 17.6-60.4 (NCB) 20.7-39.3 (AC) 14.2-29.8 (NAC)	NR	NR	18
Elias/2014/France[53]	91.4	54	36.5	4.2	17	41	NR	NR	NR	62.4 (55.6-77.6)
Frøysnes/2016/Norway[114]	93	65	36	0	15.5	47	42-52	NR	10 (7-12)	45 (35-55)
Hamilton/2011/Canada[115]	79	38	34	NR	NR	27	0-87	NR	9 (0-87) 3-yr 34% 5-yr 26%	28 (0-119) (all)
Hompes/2012/Belgium[116]	97.9	84	NA	0	52.1	NA	NA	NR	19.8 (RFS)	22.7 (3.2-55.7)
Passot/2016/France[117]	83	51	31	NR	30	36	NR	NR	11	NR
Prada-Villaverde/2014/Spain[118]	85	45	35	NR	NR	31.4	NR	NR	NR	NR
Quenet/2011/France[119]	92	36	44	4.1	47.2	41	32-60	NR	10.9 3-yr 15%	30.3 (2-88)

Adapted from Huang *et al*[112]. SR: Survival rate; OS: Overall survival; PFS: Progression-free survival; RFS: Recurrence-free survival; DFS: Disease-free survival; NA: Not achieved; NR: Not reported; NNT: Non-neoadjuvant therapy; NCA: Neoadjuvant chemotherapy alone; NCB: Neoadjuvant chemotherapy + bevacizumab; AC: Adjuvant chemotherapy; NAC: Non-adjuvant chemotherapy; CI: Confidence interval.

chemotherapy regimens still require further study.

IMPORTANCE AND RECOMMENDATION FOR THE FOLLOW-UP

At present, there are no shared guidelines for the ideal duration and intervals for CRC patient follow-up. The NCCN and European Society of Medical Oncology guidelines recommend abdomen and chest CT surveillance every 6 to 12 mo for 3 to 5 years. The American Society of Clinical Oncology (ASCO) and American Cancer Society

recommend CT every 12 mo for 5 years.

Ultrasonography provides no additional advantages for the early detection of CRC recurrence[85]. Colonoscopy is recommended at approximately 1 year after the resection (or at 3-6 mo if not performed preoperatively because of emergency surgery), then at the third year, and every 5 years thereafter. Additionally, 18FDG-PET/CT was proposed as a complementary tool for prompt detection of asymptomatic recurrence, but consecutive studies have shown additional costs with no particular benefit for resectable disease and, hence, no improvement of overall survival[86]. Currently, it is only advisable for patients with tumor marker elevation without other evidence of disease or for those in whom recurrences are suspected with normal serum marker levels[87].

Several studies have focused on the role of an intensive follow-up after curative surgery for CRC, combining imaging resources plus CEA level screening. The global results report an improved rate of recurrence detection that could be treated by intentionally curative iterative surgery[88]. However, these early findings of metachronous disease do not seem to correspond to better results on overall survival, independent of the stage at diagnosis[89-92]. No advantage has been observed in the CEA and CT combination, and any strategy has proven a better survival advantage over a symptom-based approach[93]. Peculiarly, these findings are not consistent with the early treatment of recurrences found in rectal cancer, below the peritoneal reflection, which seem to benefit from a more intensive follow-up in pursuit of better survival[90].

Despite the limitations and bias reported in these studies, the optimal follow-up regimen for CRC patients of any stage remains controversial, and care should be taken concerning unnecessary radiation exposure of the patients and the cost-effectiveness of overly intense schedules. Notwithstanding, as radical surgery is the only actual curative treatment for colon cancer, intensive surveillance can be justified in high-risk patients, while quality evidence is achieved.

CURRENT AND FUTURE LINES OF RESEARCH

Prophylactic approach to peritoneal metastases from CRC

As mentioned before, knowing the risk factors for peritoneal spread (Table 1) allows proactive strategies for patients with high risk of developing PM to be value to set the benefit of a radical resection of located disease over the morbidity added to a patient without objective tumor spread (for example, in cases of pT4 colon cancer, which can develop peritoneal recurrence at rates of 30%-40%)

Second look surgery and prophylactic HIPEC

Second-look evaluation has always gone together with a close follow-up by CT of the chest, abdomen and pelvis, colonoscopies and CEA level surveillance. Although combined with adjuvant chemotherapy treatment, the results of the different follow-up protocols and guidelines are still heterogeneous, and the optimal management remains controversial.

Some studies have found encouraging survival outcomes for proactive strategies, such as the second-look approach[94,95], prophylactic resection of target organs for peritoneal implants during the first surgery (omentectomy, appendectomy, hepatic round ligament resection and bilateral adnexectomy)[96] or prophylactic HIPEC administration in locally advanced tumors without peritoneal carcinomatosis[97-99].

Several phase III trials are currently ongoing for the evaluation of the influence of prophylactic HIPEC in high-risk patients[100] (Table 4). For example, the ProphyloCHIP trial led by Elias has already reported some preliminary data showing no morbidity increased on patients who received second-look surgery, but no advantages in 3-year disease-free survival or overall survival have been found over classical follow-up[101]. The initial results from the COLOPEC trial, presented at ASCO 2019, do not show adjuvant HIPEC to improved 18 mo over adjuvant systemic chemotherapy on PM-free survival for high-risk patients[102].

The role of laparoscopy in the staging and second-look for peritoneal disease has been shown as a useful and feasible tool, even in previously laparotomized patients for restaging. Some experienced centers even use laparoscopy as a routine step for all the elective cytoreductive surgeries to prevent patients from an unnecessary xypho-pubic incision. Recent publications highlight the relevance of wide small bowel involvement over a proper estimation of the PCI to reject CRS[103], which could enhance the value of the laparoscopic approach in this context. Although the risk factors for peritoneal recurrence are well known and firmly confirmed by numerous publications, there is currently no strong evidence of the benefit of proactive strategies

Table 4 Studies on the ClinicalTrials.gov registry investigating the role of cytoreductive surgery and hyperthermic intraperitoneal chemotherapy on high risk patients for preventing peritoneal metastases from colorectal origin

ClinicalTrials.gov ID	Phase	High risk criteria	Control *vs* Treatment arms
NCT01226394 ProphyloCHIP trial	Phase III	Complete resection of minimal abdominal synchronous metastases or perforated tumors	Surveillance *vs* second-look laparotomy with HIPEC (intraperitoneal oxaliplatin and intravenous 5-FU) after primary resection
NCT02231086 COLOPEC trial	Phase III	T4, N0-2M0 or perforated colon cancer	Adjuvant systemic therapy only *vs* adjuvant systemic therapy + HIPEC, without resection of target organs
NCT02974556 PROMENADE trial	Phase III	T3-T4 tumors	Resection of target organs for peritoneal implants, plus HIPEC with oxaliplatin and concomitant *i.v.*, 5-fluorouracil/leucovorin
NCT02179489	Phase III	T4M0 and complete resection of minimal abdominal synchronous metastases or perforated tumors	Surgery *vs* surgery and HIPEC with mitomycin C (without preventive excision of target organs)
NCT02965248 APEC trial	Phase II	T4NanyM0 and T3-NanyM0 + mucinous or signet ring cells histology	Adjuvant systemic chemotherapy alone (arm A) *vs* systemic chemo + HIPEC with raltitrexed (arm B) or oxaliplatin (arm C)
NCT02614534 HIPEC T4	Phase III	Complete resection of T4a, bNanyM0	Surgery *vs* prophylactic HIPEC with mitomycin plus target organs excision
NCT03413254 COLOPEC-II	Phase III	Complete resection of T4a, bN0-2M0	Routine follow-up (arm A) *vs* a second exploratory laparoscopy (arm B) and third exploratory laparoscopy (arm C) if the work-up is negative
NCT02974556	Phase III	Complete resection of T3-4NanyM0	Systemic chemotherapy alone *vs* CRS/HIPEC with oxaliplatin and systemic therapy
NCT02758951	Phase II/III	Complete resection of colorectal tumors with ≤ 50% of signet ring cells.	Upfront CRS-HIPEC alone *vs* pre/post perioperative systemic chemotherapy and CRS-HIPEC
NCT02830139	Phase II	Complete resection of T3-4NanyM0	CRS + systemic chemotherapy *vs* CRS + HIPEC with paclitaxel + 5-FU + postoperative chemotherapy

CRS: Complete cytoreductive surgery; HIPEC: Hyperthermic intraperitoneal chemotherapy.

versus adequate surveillance for high-risk patients. We still await the results of the process phase III trials to shed light on the optimal management.

Pressurized intraperitoneal aerosol chemotherapy

Better survival results have been tried to achieve by boosting the cytotoxic effect of chemotherapy agents of HIPEC. A novel example of this effort is the development of a new drug delivery system known as pressurized intraperitoneal aerosol chemotherapy (PIPAC). In the context of experimental trials, this procedure is being used for patients with peritoneal region as only place of metastases from different cancer origins, but without indication for cytoreductive plus HIPEC surgery as no complete resection can be performed.

This laparoscopic and iterative procedure nebulizes the cytotoxic agents into the expanded peritoneal cavity and maintains a steady pressure with the aim of increasing drug penetration into the tissues with a more homogeneous distribution than with liquid chemotherapy. Theoretically, as the pressure decreases venous blood outflow, drugs would spend more time in contact with the tissues, so higher drug concentrations could be reached with lower doses (therefore, this would minimize systemic toxicity).

In recent years, its feasibility and safety have been extensively reported, and its efficacy for a histological response and survival benefits has been described by several teams[104]. Referring to CRC, there is still little experience but encouraging results published[105], and currently, 4 prospective clinical trials are ongoing evaluating the oncological efficacy of PIPAC for selected patients who currently would be considered for palliative care (Table 5). Pursuing an optimal macroscopic cytoreduction for the best guarantee of prognosis, some trials have proposed the use of

intraoperative imaging techniques that can guide the detection of malignant lesions using tumortargeted fluorescence traces[106].

Some other trials have attempted to rescue high PCI patients for an eventual CRS surgery by using a combination of intraperitoneal plus systemic chemotherapy regimens. The aim of this bidirectional chemotherapy is to reach peritoneal implants not only from the peritoneal cavity but also from subperitoneal blood vessels[107,108].

CONCLUSION

CRS plus HIPEC combined with systemic modern chemotherapy is feasible for the management for PM of CR origin most widely accepted by experts, as accumulative evidence suggests that it improves recurrences as well as overall and peritoneal disease-free survival. Because of the lack of randomized clinical trials and the conflicting data on clinical efficacy, this approach remains controversial. Optimization of preoperative imaging assessment of tumor burden and molecular biology categorization are two promising approaches for better individualized treatment. In referral centers, the morbidity and mortality associated with this procedure do not seem to be higher than other major abdominal surgeries. Iterative CRS for local recurrences has proven to improve overall survival without adding a significant morbidity. It is paramount that patients with peritoneal carcinomatosis continue to be referred to experienced centers that can offer a multidisciplinary, tailored evaluation and high-quality surgery. As complete CRS has confidently proven to improve patient survival, future strategy targets are focused on assessing the real role of modern systemic chemotherapy and HIPEC for the treatment and prevention of PM in high-risk patients.

Table 5 Studies on the ClinicalTrials.gov registry investigating the role of pressurized intraperitoneal aerosol chemotherapy

Number Clinical Trial	Experimental therapy	Malignant origin	Cytotoxic drugs	Primary outcomes
NCT03280511 The PIPAC-OPC3 CC Trial	Exploratory Laparoscopy + biopsies + 1st PIPAC 2 mo after radical primary resection+/-adyuvant chemo + 2nd PIPAC 5 wk later	High risk colorectal cancer patients	Oxaliplatin (92 mg/m²)	Peritoneal recurrence
NCT03246321 CRC-PIPAC	ePIPAC + leuvocorin + 5-FU iv	Colorectal / appendiceal carcinomas	Oxaliplatin (92 mg/m²)	Major toxicity
NCT03210298	PIPAC/PITAC	Peritoneal metastasis of various origins	Depends on tumor origin	Overall survival
NCT02604784 PI-CaP	PIPAC fixed repeated dose *vs* PIPAC increasing single dose	Gastric, colorectal and ovarian cancers or primary peritoneal tumors	Cisplatin + doxorubicin or oxaliplatin (92 mg/m²)	Overall response rate

PIPAC: Pressurized intraperitoneal aerosol chemotherapy; ePIPAC: Electrostatic pressurised intraperitoneal aerosol chemotherapy.

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REVIEW

Eosinophils in the gastrointestinal tract and their role in the pathogenesis of major colorectal disorders

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Abstract

Eosinophils are currently regarded as versatile mobile cells controlling and regulating multiple biological pathways and responses in health and disease. These cells store in their specific granules numerous biologically active substances (cytotoxic cationic proteins, cytokines, growth factors, chemokines, enzymes) ready for rapid release. The human gut is the main destination of eosinophils that are produced and matured in the bone marrow and then transferred to target tissues through the circulation. In health the most important functions of gut-residing eosinophils comprise their participation in the maintenance of the protective mucosal barrier and interactions with other immune cells in providing immunity to microbiota of the gut lumen. Eosinophils are closely involved in the development of inflammatory bowel disease (IBD), when their cytotoxic granule proteins cause damage to host tissues. However, their roles in Crohn's disease and ulcerative colitis appear to follow different immune response patterns. Eosinophils in IBD are especially important in altering the structure and protective functions of the mucosal barrier and modulating massive neutrophil influx to the *lamina propria* followed by transepithelial migration to colorectal mucus. IBD-associated inflammatory process involving eosinophils then appears to expand to the mucus overlaying the internal gut surface. The author hypothesises that immune responses within colorectal mucus as well as ETosis exerted by both neutrophils and eosinophils on the both sides of the colonic epithelial barrier act as additional pathogenetic factors in IBD. Literature analysis also shows an association between elevated eosinophil levels and better colorectal cancer (CRC) prognosis, but mechanisms behind this effect remain to be elucidated. In conclusion, the author emphasises the importance of investigating colorectal mucus in IBD and CRC patients as a previously unexplored milieu of disease-related inflammatory responses.

Key words: Eosinophils; Eosinophilopoiesis; Gut *lamina propria*; Colorectal mucus; Normal human small intestine; Normal human colon; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Colorectal cancer

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Core tip: Eosinophils are multifunctional granulocytes possessing readily releasable stores of cytotoxic proteins, regulatory cytokines and chemokines in their specific granules. In health eosinophils reside in the gut, exerting homeostatic functions including protective mucosal barrier integrity maintenance and contribution to gut-associated immunity. Eosinophils are important players in inflammatory bowel disease pathogenesis (both Crohn's disease and ulcerative colitis). These cells are also associated with a favourable prognosis in colorectal cancer, however mechanisms of this association remain obscure. The author presents a comprehensive analysis of the current literature on eosinophils in the gut and highlights the importance of poorly investigated immune responses occurring within colorectal mucus.

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INTRODUCTION

The eosinophil was first described in the middle of XIX century when these granulocytes displaying a strong affinity to acidophilic dyes producing red staining of their granules were first identified. The honour of discovering and characterising eosinophils belongs to Paul Ehrlich, who also made first assumptions regarding their biological significance[1]. For many decades eosinophils were regarded as terminally differentiated end-stage cells of innate immunity exerting cytotoxic effector defence against parasitic helminths, but also capable of causing damage to host tissues in allergic conditions[2-5]. That simplistic paradigm had to be fundamentally revised once recent research advances revealed numerous previously unknown eosinophil functions, comprising inflammation control, epithelial barrier maintenance, participation in tissue remodelling and linking innate and adaptive immunity[2-5]. Nonetheless, the whole range of roles played by these granulocytes in health and disease remains to be elucidated.

This review focuses on eosinophil action in the distal gastrointestinal tract, both in the normal physiological conditions and in pathology, especially in the context of inflammatory bowel disease (IBD) and colorectal cancer (CRC). The declared task looks timely since eosinophils, despite being recognised as important players in both gut homeostasis maintenance and colorectal disorder pathogenesis, are often overlooked when disease mechanisms are considered. It is remarkable that none of recent comprehensive reviews on the pathogenesis of either IBD[6-10] or CRC[11-15] even mentions possible eosinophil involvement in these complex processes. The author decided to focus on analysing literature describing research in humans, but experimental modelling, especially the use of genetically modified mice is one of the key sources of new information in the field. For this reason, selected experimental studies are considered as well, but caution is always required when results obtained in murine models are extrapolated to humans.

EOSINOPHIL DEVELOPMENT AND MATURATION

Eosinophils are continuously generated in the bone marrow from pluripotent $CD34^+$ stem cells, and it was presumed that their development proceeds through the granulocyte/macrophage progenitor (GMP) in mice and the common myeloid progenitor (CMP) in humans[2,16-19]. However, using a mouse model, Drissen *et al*[20] have recently identified a distinct myeloid differentiation pathway characterised by GATA1 expression and giving rise to eosinophils, mast cells, megakaryocytes and erythroid cells, but not to monocytes, neutrophils and lymphocytes. In any case, it is generally accepted that the control of eosinophilopoiesis is exerted on the one hand by transcription factors produced internally by the developing cells of the eosinophil lineage, but on the other hand by cytokines secreted predominantly by other immune cells (Figure 1).

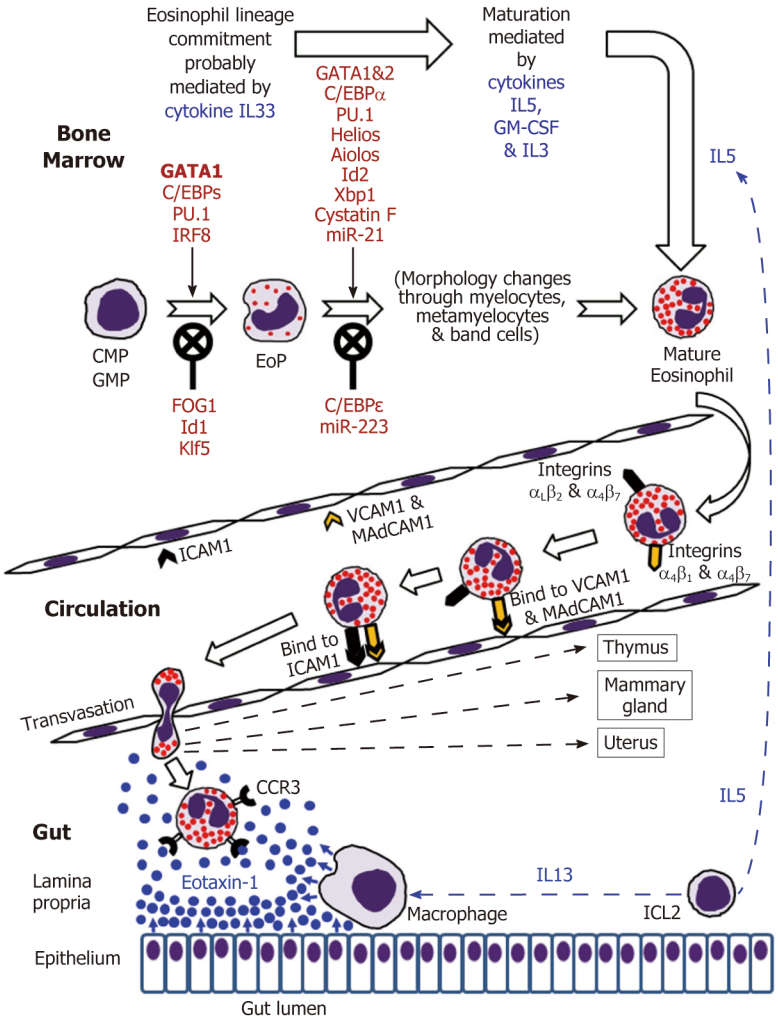

Figure 1 Eosinophil development in the bone marrow followed by mature eosinophil appearance in the circulation and eventual migration to the gut in the normal conditions.

The analysis of cell transcriptome changes during eosinophil lineage commitment followed by maturation has revealed a marked development-related increase in the number of expressed genes[21]. Early stages of this process appear to be regulated by cytokine IL33[22] and are driven at the cellular level by a few transcription factors, GATA1 being essential for eosinophil lineage commitment. Figure 1 illustrates the necessity of the interplay between pro-differentiation transcription factors including GATA1 (the key element), C/EBPs, PU.1 and IRF-8[16-19] and inhibitory influences of FOG1, Id1 and Klf5 in generating eosinophil-restricted precursor (EoP) cells[16-19,23,24]. Importantly, the acquisition of surface receptor IL5Rα occurs at the EoP stage[25], thus making EoP cells responsive to the external signalling by IL5, an eosinophil-targeting cytokine secreted by type 2 innate immune cells (ILC2)[26]. Indeed, IL5, IL3 and GM-CSF are recognised as the three cytokines regulating eosinophil proliferation, maturation and functional activities in homeostasis and pathology[2,16,18]. Their stimulating effect during eosinophil maturation is modulated by influences of several transcription factors as well as protease activity regulator cystatin F and regulatory miRNAs (Figure 1)[16-19,21,23,27,28]. It is notable that terminally differentiated mature eosinophils produced in the bone marrow have fully formed specific granules essential for the functions of these cells (see below).

DISTINCTIVE FEATURES OF MATURE EOSINOPHILS

Morphological characteristics

Mature human eosinophils can be easily morphologically distinguished by their bilobed nuclei and the presence of specific cytoplasmic granules stained red by eosin. These specific (also called crystalloid, secretory or "secondary") granules are unique

organelles that consist of a dense central crystalloid core and an outer matrix surrounded by a trilaminar membrane[4,29,30]. Although earlier publications sometimes mention the existence of both large "primary" granules lacking the crystalloid core and rich in Charcot-Leyden crystal protein and "small" granules, recent studies suggest that the coreless large granules are simply immature specific granules, whereas the "small" granules are membrane-bound vesiculotubular structures associated with eosinophil secretory activity[31]. Thus, there is only one granule type present in human eosinophils, and these specific granules store pre-formed biologically active substances comprising cytotoxic cationic proteins, cytokines, growth factors, chemokines and enzymes[4,32,33]. Other eosinophil-specific organelles are lipid bodies[34] charged with the synthesis of lipid mediators of inflammation (cysteinyl leukotrienes, thromboxanes and prostaglandins) and pleiomorphic vesiculotubular carriers (eosinophil sombrero vesicles)[35]. Like all somatic cells, eosinophils also have mitochondria, endoplasmic reticulum and Golgi bodies.

Arsenal of secretory substances and cell surface markers expressed by eosinophils

Specific granule formation and maturation associated with the accumulation of cationic granule proteins is essential for eosinophil development, function and survival[4,5,32,33]. Table 1 presents key biomolecules secreted by human eosinophils, and it is evident that, in addition to the well-known cationic proteins, these cells contain pre-formed stores of numerous regulatory molecules including cytokines, chemokines, growth factors and enzymes. Studies in murine models also demonstrated that parallel *de novo* synthesis of cytokines occurs in mature eosinophils[40-42], however the relationship between pre-formed and *de novo* synthesised regulatory factors remains to be elucidated.

In addition to secretory substances, eosinophils express a considerable number of surface markers comprising receptors for adhesion molecules, cytokines, chemokines, growth factors, lipid mediators as well as pattern recognition receptors (PRRs) and Fc receptors. Table 2 lists hitherto identified eosinophil surface markers. Notably, stores of receptor molecules were also identified within the specific granules[55], and membrane receptors for cytokines and chemokines located on the surface of these granules enable them to act as receptor-mediated secretory organelles even extracellularly[56,57]. The abundance of receptors and cell surface-associated molecules defines eosinophil ability of participating in an extremely wide range of physiological and pathological processes. More details on eosinophil surface markers can be found in a few recent reviews (Rosenberg *et al*[5], Ravin *et al*[43] and Gangwar *et al*[44]).

Types of secretion/degranulation observed in human eosinophils in relation to their functional activities

The current view on the versatility of eosinophil action in health and disease was concisely formulated by Lee *et al*[3] in their LIAR (Local Immunity And/or Remodelling/Repair) hypothesis. Expanding this notion, eosinophil functions can be classified into the following four categories: (1) Terminal effector functions; (2) Maintaining homeostasis and supporting tissue repair/remodelling; (3) Immunomodulatory role; and (4) Cooperative interactions with other immune cells[4]. Essentially, all functional activities of eosinophils in both normal and pathological conditions are exerted through the secretion of their specific products. Although classical or compound exocytosis implying granule fusion with the plasma membrane followed by extracellular release of entire granule contents[58] may be occasionally employed by eosinophils attacking multicellular helminths[33,59], this secretory mechanism is rarely observed during inflammatory and allergic responses, when immediate but selective release of pre-formed granule-stored factors is required. Extensive investigation of eosinophil degranulation modes has shown that either piecemeal degranulation (PMD) or cytolysis followed by whole granule release clearly prevail in most situations associated with human disease[33,59,60].

PMD is characterised by stimulus-dependent (receptor-mediated) differential packaging of selected granule-derived proteins into secretory vesicles that are then transported to the cell surface and expelled through it. During this process specific granules are partially emptied but otherwise remain intact[60]. Being highly selective and rapid, PMD is typically employed for cytokine secretion by eosinophils. It is the most common secretion type observed in the context of inflammation and allergy in humans[5,33,59,60].

Recent reports imply that cytolysis or primary lysis of human eosinophils[61] is the second most frequently observed degranulation mode in inflammatory and allergic conditions[33,60,61]. Cytolysis is now regarded as a regulated mechanism of rapid cell death (distinct from apoptosis and necrosis) that is characterised by chromatin decondensation and dissolution of nuclear and plasma membranes as well as the release

Table 1 Secretory substances produced by eosinophils

Substance	Characteristics	Pre-prodiced or *de novo* synthesysed	Main functions	Ref.
Located within the core of specific (crystalloid) granules				
Major basic proteins 1 and 2 (MBP1 and MBP2)	Small highly cationic proteins (MBP2 is less basic)	Pre-produced	Highly cytotoxic to host cells, antihelminthic, antibacterial (MBP1 is more potent and associated with EETosis)	[2,4,32,36]
Located within the matrix of specific (crystalloid) granules				
Eosinophil cationic protein (ECP)	Small highly cationic protein with a weak ribonuclease activity (ribonuclease 3)	Pre-produced	Highly cytotoxic to host cells, neurotoxic, antihelminthic, antibacterial, antiviral, associated with EETosis	[2,4,32,36]
Eosinophil-derived neurotoxin	Small basic (less cationic than MBP1 and ECP) protein with a ribonuclease activity (ribonuclease 2)	Pre-produced	Cytotoxic to host cells, neurotoxic, strongly antiviral, antibacterial,	[2,4,32,36]
Eosinophil peroxidase	Highly cationic heme-containing haloperoxidase	Pre-produced	Generates ROS exerting potent antibacterial and antihelminthic effects	[2,4,32,36]
Charcot-Leyden crystal protein (CLC, galectin-10)	Small slightly acidic protein	Probably pre-produced	Unclear, but involvement EETosis and a role in interactions between eosinophils and T-cells are suggested	[31,32,36-38]
Most likely stored within specific granules, but some may be synthesised *de novo* in the cytoplasm				
IL1β, IL2, IL3, IL4, IL5, IL6, IL10, IL11, IL13, IL16, IL18, IL25 (IL17E), IFNγ, GM-CSF, TGFα, TNFα, TNFβ, leukaemia inhibitory factor	Cytokines	Mostly pre-produced; some may be *de novo* synthesised	A wide range of signaling and regulatory functions	[4,5,32,39]
HB-EGF-LBP, NGF, PDGF, SCF, EGF, VEGF, APRIL	Growth factors	Pre-produced or *de novo* synthesised	Signalling functions related to cell proliferation and differentiation	[4,5,32]
CCL3, CCL5, CCL6, CCL7, CCL8, CCL9, CCL11 (eotaxin-1), CCL13, CXCL1, CXCL8, CXCL10, CXCL12	Chemokines	Pre-produced or *de novo* synthesised	Cell migration regulation	[4,5,32,39]
Matrix metalloproteinases MMP9 and MMP17, acid phosphatase, collagenase, arylsulfatase B, histaminase, phospholipase D, catalase, non-specific esterases	Enzymes	Pre-produced or *de novo* synthesised	Inflammation-related effector functions including cytotoxicity, extracellular matrix modification and phagocytosis	[4,5,32]
Produced within the lipid bodies				
Leukotrienes C4, D4 and E4, Thromboxane B2, Prostaglandins E1 and E2, 15-hydroxyeicosatetranoeic acid, platelet-activating factor	Lipid signalling factors, mostly eicosanoids	*De novo* synthesised	A broad range of effects in inflammatory and allergic responses	[4,5,34]

of intact specific granules retaining their functionality[33,56,57,59]. It was later demonstrated that eosinophil cytolysis is often accompanied by the formation of "extracellular traps", *i.e.*, web-like nets composed of extruded histone-coated strands of DNA and specific granules released from the same lysed cells[62-64]. The latter phenomenon is similar to the formation of bactericidal NETs (neutrophil extracellular traps) by neutrophils first described in 2004 by Brinkmann *et al*[65] and often defined as a unique form of cell death initially called NETosis[66]. It is, however, more appropriate to call this phenomenon ETosis since it is not neutrophil-specific and was observed in eosinophils[62-64], mast cells[67], basophils[68], monocytes[69], macrophages[70] and even B and T lymphocytes[71]. Moreover, an alternative, "non-lethal", mechanism of extracellular DNA trap generation by eosinophils was described by Yousefi *et al*[72], who observed a catapult-like release of mitochondrial DNA from eosinophils remaining alive. The released DNA formed extracellular traps that also contained eosinophil granule proteins apparently secreted through PMD[72]. In the context of eosinophil effector functions, the association of released (by either mechanism) DNA

Table 2 Cell surface markers expressed by eosinophils

Group of surface markers	Markers identified for eosinophils	Ref.
Cytokine and growth factor receptors	IL-2R, IL-3R, IL-4R, IL-5R, IL-9R, IL-10R, IL-13R, IL-17R, IL-23R, IL-27-R, IL-31R, IL-33R (ST2), TSLPR, GM-CSFR, KIT, IFNγR, TGFβR	[5,43-45]
Chemokine and chemoattractant receptors	CCR1, CCR3, CCR4, CCR5, CCR6, CCR8, CCR9, CXCR2, CXCR3, CXCR4, FPR1, FPR2, C3aR, C5aR	[5,43,44,46]
Lipid mediator receptors	Platelet-activating factor receptor, DP1 prostaglandin receptor, DP2 prostaglandin receptor (CRTH2), EP4 prostaglandin receptor, E2 prostaglandin receptor, Leukotriene B4 receptor, Lysophosphatidylserine receptor P2Y10, S1P receptors	[5,43,44,47]
Fc receptors	FcαR, FcγRII, FcγRIII, FcεRI, FcεRII	[5,43,44]
Adhesion molecule receptors	Integrin $\alpha_L\beta_2$ (LFA1), Integrin $\alpha_M\beta_2$ (CR3), Integrin $\alpha_X\beta_2$ (CR4), Integrin $\alpha_4\beta_1$ (VLA4), Integrin $\alpha_6\beta_1$, Integrin $\alpha_D\beta_2$, Integrin $\alpha_4\beta_7$ (LPAM), cell surface adhesion receptor CD44, CD62L (L-selectin), PSGL1 (P-selectin lipoprotein ligand), CD34, CD244 (2B4)	[5,43,44,48,49]
Pattern recognition receptors	TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR9, TLR10, NOD1, NOD2, RIG-1, RAGE	[5,43,44]
Other receptors and surface markers	PIRB, SIGLECs, SIRPα (shown for mice), LAIR1, Cannabinoid receptor CB2, Kinin B1 and B2 receptors, Histamine receptors, PAR1 or PAR2, CD80 or CD86, CD48, CD300 receptors (a and f), MHC class II	[5,43,44,50-53]

fibres and functional specific granules (or their proteins) certainly provides a formidable defensive weapon that can be effectively used against infective agents (bacteria, viruses, fungi) or multicellular parasites (helminths)[64]. However, extracellular traps generated by eosinophils are highly cytotoxic and can damage host tissues. Both defensive and host-damaging effects of eosinophils are especially important for barrier tissues including the gut and will be discussed in more detail in further sections of this review.

MIGRATION OF MATURE EOSINOPHILS TO THE GUT

According to generally accepted views, upon maturation in the bone marrow eosinophils enter the circulation and migrate to the gastrointestinal tract, their accumulation in the gut mucosa commencing during embryonal development[73,74].

IL5 and possibly GM-CSF stimulate the release of eosinophils from the bone marrow into the circulation[2,16,32]. Mouse eosinophils can stay in circulation for up to 36 h[75], and this estimate has later been shown to be close to their average 25-h intravascular presence in humans[76]. Eosinophil trafficking from the circulation to the gut and other peripheral target tissues (such as thymus, uterus and mammary gland in the normal conditions) is a complex multi-step sequence of events. As for all leukocytes, it comprises tethering, rolling, adhesion and transendothelial migration followed by polarisation and amoeboid movement in the interstitial space[73,77-81]. Alongside chemokine and cytokine signalling, the interaction between adhesion molecule receptors expressed by eosinophils and corresponding adhesion proteins of endothelial cells is crucially important for efficient eosinophil trafficking. Although leukocyte extravasation is better studied in inflammatory conditions, it can be assumed that in health the initial adherence (tethering) of eosinophils to endothelial cells and the initiation of rolling motion are selectin-dependent, whereas further rolling, firm adhesion and transendothelial migration are mediated by integrins[77-79]. In the context of eosinophil homing in the intestinal *lamina propria* (schematically shown in Figure 1), binding of the integrins $\alpha_4\beta_7$ and $\alpha_4\beta_1$ expressed by eosinophils to endothelial adhesion molecules MAdCAM1 and VCAM1 respectively is especially important, and interactions of $\alpha_L\beta_2$ and $\alpha_M\beta_2$ with ICAM1 appear to lead to the eventual transmigration from capillaries[48,82,83]. Further interstitial migration of eosinophils to their destination in the *lamina propria* is believed to be governed mostly by chemokine eotaxin-1 interacting with its CCR3 receptor on the surface of eosinophils[2,74]. Eotaxin-2 and eotaxin-3 may also contribute to the chemotaxis, but

appear to be less eosinophil-specific[2,74]. In the human colon eotaxin-1 concentration gradient directing eosinophil migration depends on the secretion of this chemokine by intestinal macrophages and epithelial cells[84-86], while IL13 produced by ILC2 cells[26] stimulates macrophage eotaxin-1 expression (Figure 1).

EOSINOPHILS IN HEALTHY GASTROINTESTINAL TRACT

Mouse experiments demonstrated that eosinophil homing in the gut occurs in the foetal life, *i.e.*, independently of later intestinal colonisation by microbiota[73]. In the normal human gastrointestinal tract the presence of eosinophils increases in the distal direction (oesophagus < stomach < small intestine < colon) reaching its peak in the caecum and ascending colon[87-89]. As already mentioned, in the normal gut eosinophils are primarily located in the *lamina propria* of the mucosa rather than in the surface epithelium[82,90].

Unfortunately, functions of human gastrointestinal eosinophils in health remain poorly investigated, mouse models being the main source of available information. Still, recent progress in this complex field allows making some generalisations.

Eosinophil presence in the gut was previously believed to simply constitute an effector element of the innate host defensive barrier, but it is now becoming clear that resident intestinal eosinophils continuously monitor and modulate complex immune responses and tissue remodelling throughout the huge surface of the gastrointestinal tract[3]. It is remarkable that, unlike their circulating counterparts, gastrointestinal eosinophils exhibit an activated phenotype suggesting permanent functional activity[4,91]. Participation in the following physiological mechanisms is currently attributed to these gut-dwelling cells: (1) Maintenance of gastrointestinal mucosal barrier function; (2) Provision of immunity to pathogens present in the gut lumen; (3) Interactions with the enteric nervous system; (4) Linking innate and adaptive immunity[4]. These functions are discussed in more detail hereafter.

Eosinophil participation in the maintenance of gastrointestinal mucosal barrier function

The intestinal epithelium acts as a uniquely important body interface with the environment. In addition to its physical barrier function that prevents underlying tissues from contacting harmful microbiota and dietary antigens of the gut lumen, this epithelium regulates the selective absorption of nutrients, water and electrolytes[92]. The epithelial surface throughout the gut is covered by the glycocalyx of enterocytes and colonocytes[93] and abundant mucus rich in mucin 2 (MUC2) produced by goblet cells. The utmost importance of this mucus became evident only recently, and it is now established that the structure of the mucus layer throughout the intestine is not uniform. The small intestine has a single and relatively loose layer of mucus populated by microbiota, whereas the colon has a two-layered system with a dense inner layer firmly attached to the mucosal surface and an outer layer permeable for bacteria (Figure 2)[94,95]. It is remarkable that this structure essentially excludes any direct contact of bacteria with the colonic epithelium. The protective role of the mucus is further enhanced by the presence of antibacterial substances, such as α-defensins and lysozyme secreted by Paneth cells of small intestinal crypts[96] and IgA produced by plasma cells of the *lamina propria*[97,98].

Interestingly, it has recently been shown that intestinal mucus layer maintenance depends on eosinophil presence in the *lamina propria* since eosinophil-deficient mice had significantly decreased numbers of mucus-secreting goblet cells in the small intestine[98]. This is not surprising since eosinophils are known to directly induce mucin production in airway epithelial cells by activating EGFR cascade[99]. Moreover, although possible interactions between eosinophils and Paneth cells remain obscure, the transcription factor Xbp1 is important for the development of the both cell types[27], and, like Paneth cells, human eosinophils were demonstrated to produce α-defensins[100]. It was also reported that eosinophils are required for maintaining mucosal IgA production by plasma cells in the *lamina propria*[97,98,101], probably through mechanisms involving IL1β[98] and TGFβ[97,100] signalling.

Taken together, this information suggests that eosinophils are intimately involved in the maintenance of the protective intestinal mucus in the normal conditions.

Eosinophil participation in the provision of immunity to pathogens in the gut lumen

The immune system of the gut is an extremely complex entity, analysis of which is beyond the scope of this review, but eosinophil impacts are highlighted herein. It is obvious that in the normal homeostatic conditions immune surveillance focused on sampling and assessing luminal antigens occasionally contacting the mucosal surface

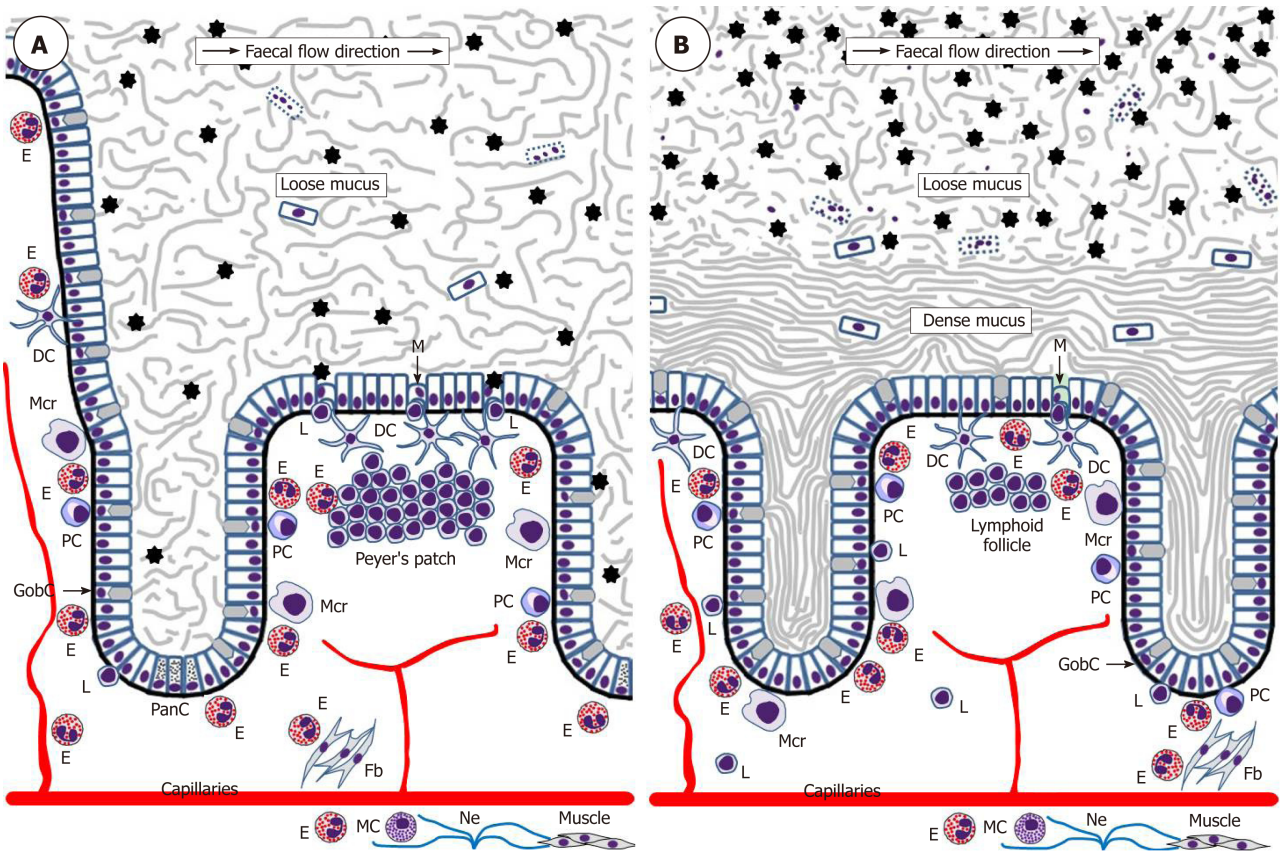

Figure 2 Schematic representation of eosinophil interactions with other cells and tissues in the small intestine (A) and colon (B). Black asterisks indicate gut microbiota. Although one M-cell is shown in the colon, little is known about the presence of M-cells in healthy human colon.DC: Dendritic lells; E: Eosinophils; Fb: Fibroblasts; GobC: Goblet cells; L: Lymphocytes; M: M-cells; Mcr: Macrophages; Ne: Nerves; PanC: Paneth cells; PC: Plasma cells.

is of utmost importance. The gut-associated lymphoid tissue (GALT) is believed to be responsible for luminal antigen sampling. In the small intestine this function is primarily associated with microfold (M) cells[102-104] located in the epithelium overlaying Peyer's patches (Figure 2) and capable of communicating with immune cells comprising B lymphocytes, T lymphocytes, macrophages and dendritic cells. M-cells are also believed to be present in the epithelium overlaying lymphoid follicles in the colon[103], but their location and functions in the normal human colon remain very poorly investigated. M-cells exert luminal antigen transcytosis followed by antigen transfer to the relevant immune cells of the *lamina propria*, but alternative antigen capture mechanisms appear to exist as well. Dendritic cells were shown to sample bacterial antigens by extending their dendrites to penetrate the epithelium and reach the lumen[105,106]. In addition, goblet cells can deliver low molecular weight intestinal antigens to the underlying dendritic cells of the *lamina propria*[107]. The latter mechanism shown for the normal small intestine[107] may be especially important for the colon, where it becomes activated only when mucus barrier, luminal microbiota balance or microbial sensing by goblet cells are disturbed[108]. Given that the stratified mucus layer securely protects colonic epithelium from any contact with gut contents in the normal conditions, it is likely that immune cells located in the colonic *lamina propria* may be completely unaware of luminal antigens until the protective barrier is damaged[108]. Although the role of eosinophils in luminal antigen recognition and presentation at baseline remains poorly investigated, it should be noted that these granulocytes promote the development of Peyer's patches[98] and are known to be closely involved in regulating dendritic cell activation and migration[109]. In addition, intestinal eosinophils can express antigen presentation-associated markers, including MHCII and CD80[91] as well as activating receptor FcγRIII[110]. Their possible antigen-presenting function has not been demonstrated in the normal gut and remains to be investigated.

Eosinophil role in the provision of interactions with the enteric nervous system

Eosinophil influences on the nervous system were extensively studied in the context of asthma and allergic respiratory inflammation[111,112]. It is also known that human eosinophils can produce nerve growth factor in abundance[113]. Eosinophil-nerve

interactions in the gut were demonstrated experimentally[114], and accumulating evidence indicates that eosinophil impact on the enteric nervous system may often be exerted through mast cells residing in the *lamina propria*[115]. Indeed, eosinophils and mast cells are often found in close proximity to each other. Physical interaction between them can induce a hyperactivation state accompanied by soluble mediator release[116,117]. Mast cells and eosinophils in the gut are often located near sensory nerve fibres and are known to be involved in the pathogenesis of functional gastrointestinal disorders accompanied by motility changes, hyperalgesia and diarrhoea[115,118]. Concerted action of eosinophils and mast cells can produce multiple effects in addition to interactions with the nervous system, being among major factors in allergy and responses to infections[119], which are beyond the scope of this review.

Eosinophil role in linking innate and adaptive immunity in the intestine

It is now recognised that eosinophils can modulate T cell-mediated immune responses, owing to their ability to rapidly produce cytokines, chemokines and growth factors[3-5], but little is known about their regulatory functions in the normal intestine. Although eosinophils are able to secrete cytokines associated with both Th1 and Th2 cells[86], in the gut they appear to be primarily associated with Th2 immunity, being producers of Th2-inducing cytokines IL4 and IL13[120]. It is remarkable that IL13 was detectable in a considerable fraction of these cells in the normal human duodenal *lamina propria*[121]. Recent demonstration of significantly increased Th1 cell presence in the gut of eosinophil-deficient transgenic mice corroborates organ-specific Th1 immunity suppression by these granulocytes[122].

It is assumed that eosinophils may regulate the magnitude and Th2 polarisation of immune responses through interactions with B and T lymphocytes[86]. However, there are many unanswered questions regarding types of cell-mediated immune responses and corresponding cytokine profiles, and a view advocating the existence of three rather than two types of effector immunity has recently emerged[123]. Only further intense research in this dynamic area will lead to better understanding of eosinophil interactions with other immune cells in the human gut.

Clearly, the four topics highlighted above do not entirely cover all activities of intestinal eosinophils. For instance, experimental studies have revealed eosinophil influences on smooth muscle cells[74,124], fibroblasts[74,125] and capillary endothelium[74], but these roles remain to be investigated for the normal human gut.

EOSINOPHIL IMPACT IN THE PATHOGENESIS OF MAJOR COLORECTAL DISEASES (IBD and CRC)

The author opted not to address eosinophil-associated gastrointestinal disorders (EGID) comprising eosinophilic oesophagitis, eosinophilic gastritis, eosinophilic enteritis and eosinophilic colitis as well as gastrointestinal manifestations of hypereosinophilic syndromes. These relatively rare conditions were extensively reviewed elsewhere[83,86,126,127]. The concluding part of this review is focused on two major colorectal diseases: IBD and CRC.

Eosinophils in the pathogenesis of IBD

IBD is represented mainly by two major conditions: ulcerative colitis (UC), which affects exclusively colonic mucosa[10], and Crohn's disease (CD), a transmural asymmetrical inflammation that can involve the entire gastrointestinal tract[9]. These diseases are highly prevalent all over the world[128-131]. Both UC and CD are chronic relapsing disorders with complex and not entirely understood mechanisms of development. IBD etiopathogenesis is, however, believed to include genetic predisposition, environmental/microbial impacts, intestinal barrier dysfunction and dysregulation of the mucosal immune system of the gut[6-10,132]. The latter two pathogenetic components can certainly be influenced by eosinophils, the abundance of which, correlating with disease severity, is well documented in the gut mucosa of patients with UC[133-140] and some CD cases[135,137,141,142]. The increased presence of eosinophils in the mucosa of these patients apparently results from an enhanced production of eotaxin 1 in the *lamina propria*[84,143-145] by colonocytes[84,136], macrophages[84,145] or B lymphocytes[144]. Furthermore, Manousou *et al*[146] described an elevated expression of eotaxin receptor CCR3 in colonic biopsy samples from UC rather than CD patients. Although the authors interpreted this finding as a sign of the accumulation of CCR3-expressing T cells, it is likely that *lamina propria* eosinophils expressing the same receptor could be at least equally responsible for this UC-associated change. Conversely, serum eotaxin levels were reported to surge in active IBD[141,147,148], but the increase looked more pronounced in CD patients compared to UC

cases[147,148]. The contrast between UC and CD becomes even more evident in view of different patterns of colonic eosinophil activation described by Lampinen *et al*[84,136,137,145,170,186,188] and indicating that in disease remission activated eosinophils persisted in the *lamina propria* of UC, but not CD patients[136,137].

The observed differences in colonic eosinophil presence and activity between the two IBD types are not surprising because it is traditionally accepted that CD pathogenesis, which has a stronger genetic component[6], is dominated by Th1 cytokine profile (combined with Th17 influences)[149-152], whereas Th2 immune response tends to characterise UC[150-153]. Given recent advances in the understanding of immune response types, involved cell populations and cytokine profiles[123,154,155], this straightforward division may now look simplistic, but the association of eosinophils with Th2 immunity is well proven[120]. Therefore, their role in UC pathogenesis looks more evident and easier to explain.

Before further discussing the role of eosinophils in IBD development it will be useful to briefly address gut barrier-related aspects of disease onset. Although numerous factors admittedly contribute to IBD pathogenesis[6-8,156], the precise mechanism of disease initiation remains elusive. Multiple lines of evidence indicate that in most cases IBD can be triggered by an initial contact of the gut microbiota with the mucosal immune cells followed by the development of inadequate immune responses. It is probable that preconditions for this initial contact are associated with functional deficiencies of the protective barrier of the gut mucosa, and they are likely to differ between CD and UC. Indeed, the loose mucus layer of the small intestine may be easily penetrated by microorganisms that then directly contact the epithelium, particularly mucosal M-cells[102-104] of the ileum. Such events can be facilitated by α-defensin deficiency caused by Paneth cell dysfunction that is frequently observed in CD patients, including those genetically predisposed to the disease[157,158]. In contrast, M-cells in the normal colonic mucosa can hardly be reached by microbiota since two layers of mucus, especially the dense inner one[93-95], exclude any bacterial contact with the epithelium (Figure 2B). The protective role of the inner mucus layer is well illustrated by spontaneous colitis development in MUC2-deficient mice[159,160]. Bacterial penetration through the inner mucus layer was observed in UC patients[161], but it is not entirely clear what triggers the initial change of inner mucus layer properties. Mouse models suggest MUC2 secretion deficiency[159,160] or goblet cell depletion[162] as probable causes, and goblet cell numbers in UC patients, indeed, tend to be reduced[163]. Some authors believe that unresolved endoplasmic reticulum stress and the unfolded protein response are early events leading to goblet cell disfunction and mucus layer impairment in UC[164,165]. Altered eosinophil behaviour may well be associated with these phenomena, but this possibility remains to be investigated. Alternatively, the inner colonic mucus layer can be primarily damaged by mucus-degrading bacteria of the gut lumen[166]. The latter process may potentially be modulated by dietary factors since it was experimentally demonstrated that dietary fibre deficiency leads to switching of the gut microbiota on using mucus glycoproteins as a nutrient source and eventual erosion of the mucus barrier[167]. All the pathogenetic components discussed above may contribute to IBD initiation in humans, but further research in the area is obviously needed.

Whatever scenario causes IBD initiation, there is little doubt that bacterial antigen interaction with the gut-associated lymphoid tissue triggers complex cascades of inadequate immune responses leading to disease development. In these circumstances, activated eosinophils present in the *lamina propria* predominantly start acting as effector cells, excessive protective response of which can cause serious damage to the host through several mechanisms. Activated eosinophils accumulating in the gut of IBD patients[133-142] have an extended lifespan[168], and their degranulation leads to a massive release of both cytotoxic granule proteins and pro-inflammatory cytokines. Gut epithelium is one of the key targets of cytotoxic eosinophil proteins as it was demonstrated that MBP alters colonic epithelium barrier function[169]. Another mechanism of eosinophil contribution to colonic barrier dysfunction in UC involves muscarinic receptors expressed by these granulocytes. Wallon *et al*[170] showed that cholinergic signals received by the muscarinic receptors caused corticotropin-releasing factor (CRF) production by eosinophils, and CRF induced degranulation of neighbouring mast cells that led to an increase in mucosal barrier permeability. Concerted action of eosinophils and mast cells may have multiple endpoints in IBD as the both types of cells are important sources of cytokines comprising TNFα that, intriguingly, induces M-cell appearance in the mouse colon during inflammation[171]. Moreover, cooperation between eosinophils and mast cells was demonstrated in CD-associated fibrosis development at later stages of the disease[172]. Inflammation intensity can also be aggravated by human gut eosinophils through blocking anti-inflammatory interleukin 22 (IL22)[173] by overproducing IL22-binding protein in both CD and UC patients[174].

Interactions of gut eosinophils with other immune cells, especially lymphocytes, are very complex and poorly investigated in IBD patients. There is no room for discussing all of them here, but crosstalk between eosinophils and neutrophils deserves to be mentioned. Neutrophils are not present in the *lamina propria* in the normal conditions but are rapidly attracted there when inflammatory response develops (Figure 3). It is believed that chemokines, especially CXCL8, produced by gut epithelium in inflammation trigger neutrophil chemotaxis[175]. Interestingly, eosinophil impact in this process is now becoming evident since they can synthesise CXCL8[176]. Also, eosinophils were demonstrated to cooperate with colonic epithelium in producing a wider range of neutrophil chemoattractants[177]. In conclusion of discussing the role of *lamina propria* eosinophils in IBD it needs to be noted that the presented facts are mostly related to human disease. There is a considerable body of additional information obtained in murine models which has been reviewed elsewhere[178].

Until recently, pathogenetic mechanisms of IBD were considered only at the level of events occurring within the gut wall. However, it is now becoming evident that gut mucus layer presents another, poorly investigated but potentially highly important, battlefield for innate immune responses. Although massive neutrophil influx to the mucosa, often leading to crypt abscess formation, is recognised as a hallmark feature of IBD, the importance and mechanisms of immune cell migration through mucosal epithelia remain poorly understood and insufficiently investigated[179,180]. Transepithelial neutrophil migration involving a chain of molecular events that include initial attachment to the basal surface of epithelial cells, movement through paracellular space (passing by desmosomes, adherens junctions and tight junctions) and eventual contact with the apical membrane of the epithelium is relatively well understood[179,180]. Moreover, it is known that following transmigration neutrophils release MMP9-rich microparticles that disrupt epithelial junctions facilitating further transmigration[182]. Rapid eosinophil migration through tracheobronchial epithelium in experimental conditions was also reported[181], but remains unexplored in the gut. The process is, thus, well-defined, but the final destination of cells crossing the epithelial barrier remains obscure, being often indicated simply as "gut lumen"[179,180]. Neutrophil-associated markers of inflammation are, indeed, easily detectable in faeces of most patients with active UC and CD, as the popularity of faecal diagnostic tests, particularly stool calprotectin, proves[183,184]. Similarly, the presence of eosinophil markers in stool or colorectal perfusion fluid of IBD patients was repeatedly reported[184-188]. It is, however, apparent that any cells or biomolecules leaving gut epithelium surface should first enter mucus barrier already discussed above, and only its occasionally separated fragments can be incorporated into the faecal matter. The author of the present review previously hypothesised that colorectal mucus retains these highly informative cells and molecules and can be conveniently used for diagnosing colorectal diseases[189]. Studies of our group convincingly demonstrated the abundance of inflammatory cells, predominantly neutrophils, in colorectal mucus collected from IBD patients either intrarectally[190,191] or non-invasively[192,193]. Common eosinophil presence in this material was also noted[191,193], especially in UC patients[193], and dramatically increased EDN levels were determined[191,194], typically with higher values in UC compared to CD[194]. Detailed cytological analysis of these samples demonstrated that colorectal mucus from patients with active IBD commonly contains not only huge amounts of neutrophils, but also eosinophils, macrophages, erythrocytes as well as occasional plasma cells, lymphocytes and basophils[193]. Most of these cells are viable and functionally active as our frequent observations of phagocytosis by neutrophils and macrophages indicate[193]. Furthermore, it appears that signs of ETosis were also present in colorectal mucus from IBD patients[193]. These findings allow hypothesising that gut mucus acts in IBD as a unique additional milieu, where immune responses expand from the mucosa. It is apparent that the abundance of active cells, especially granulocytes releasing contents of their granules, considerably loosens the inner mucus layer in the colon, thus making it both permeable for gut microbiota and facilitating further immune cell transmigration and movement through the mucus. These circumstances should favour antibacterial activity of the effector cells, but extensive collateral damage of host epithelium is highly likely. ETosis exerted by neutrophils, eosinophils and other immune cells[62-72] may be especially important in this context. Figure 3 reflects the author's opinion on the extent of inflammatory process in the human gut.

Protection from invading microbiota appears to be the main biological aim of ETosis since histones enveloping released DNA are antibacterial[195], and the addition of DNA strands may increase mucus viscosity, thus mechanically compensating for MUC2 degradation. Further antibacterial action is provided by granule proteins of both neutrophils[196,197] and eosinophils (Table 1). However, ETosis in the mucus, especially combined with the release of intact eosinophil granules, can seriously

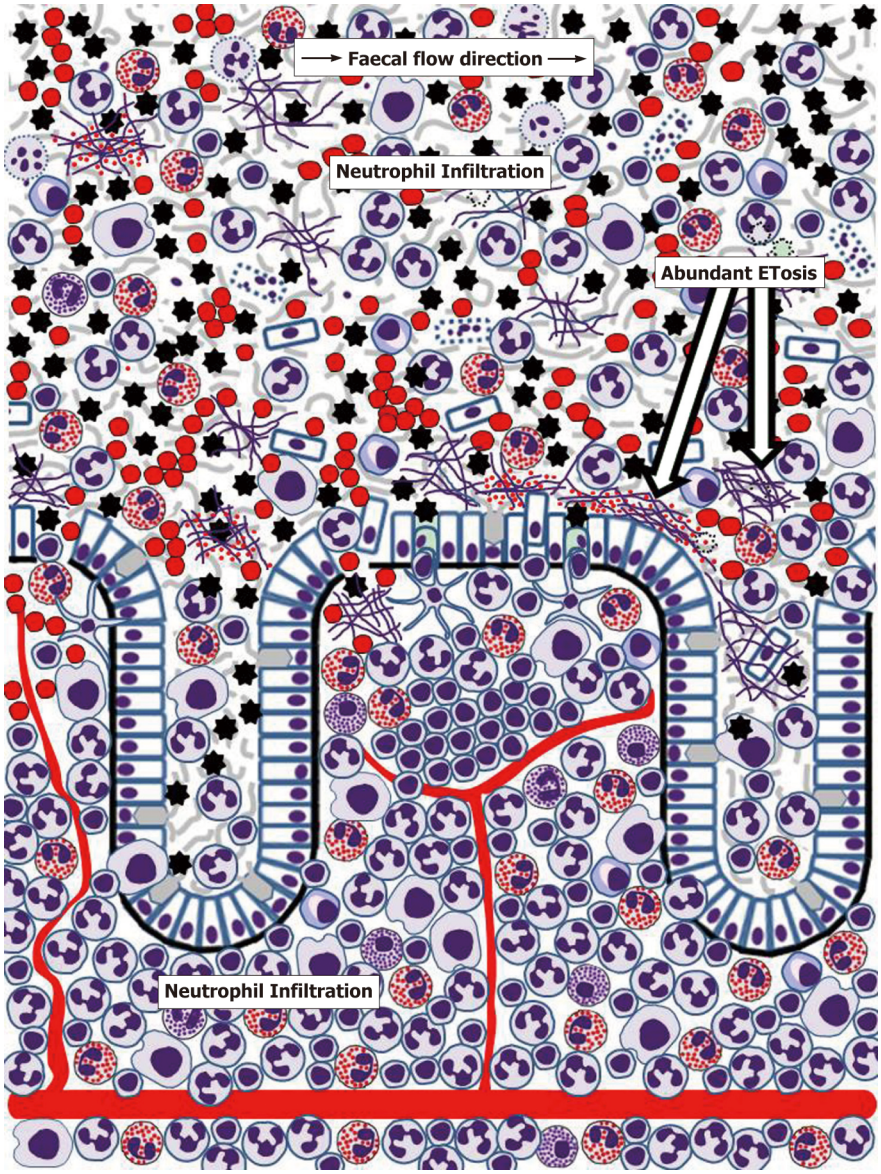

Figure 3 Schematic representation of human colonic mucosa and overlaying mucus during inflammatory bowel disease (ulcerative colitis) flare-ups. Rapid influx of neutrophils results in severe neutrophil infiltration of the *lamina propria*. Further massive transepithelial migration of neutrophils and other immune cells (especially eosinophils in ulcerative colitis) eliminates mucus layer structure, enables bacterial contact with the epithelium, causes epithelial cell death, ulcer formation and bleeding. Mucus infiltration with neutrophils and eosinophils is accompanied by abundant ETosis and release of both granule proteins and free eosinophil granules. Active inflammation also induces M-cell appearance in the epithelium overlaying lymphoid follicles[171]. Cell images correspond to those used in **Figure 2**. Erythrocytes are presented by red circles. Small red dots correspond to free eosinophil granules.

damage enterocytes or colonocytes. Eosinophil-derived extracellular DNA traps have already been shown to injure airway epithelium in chronic obstructive pulmonary disease[198] and chronic rhinosinusitis[199], where MBPs released from specific granules were especially toxic[200]. This phenomenon is still poorly investigated in relation to UC and CD, but interest in IBD-associated ETosis is emerging. In addition to our results discussed above, NET presence has been demonstrated in biopsy samples from IBD patients[201-203], notably within crypt abscesses[201], *i.e.*, beyond the mucosa. Although the significance of immune responses occurring within colorectal mucus remains to be elucidated, it is impossible to exclude that eosinophil-generated extracellular DNA threads, loaded with entrapped specific granules that release cytotoxic cationic proteins, can cause a continuing colonocyte damage leading to sustained ulceration. This so far unexplored mechanism may constitute an important pathogenetic factor in UC and, to some extent, in colonic CD. On the other hand, it should not be forgotten that transepithelial migration of immune cells is a major factor in mucosal disease resolution, as demonstrated for airway diseases[204,205].

Indeed, the presence of both inflammatory cells and biomarkers associated with them significantly decreased in colorectal mucus samples from successfully treated IBD patients[194]. It is, therefore, probable that gradual distal movement of colorectal mucus[189] creates favourable conditions for eliminating dead or obsolete immune cells from the surface of colonic epithelium if inflammation is successfully resolved.

The presented analysis of literature on eosinophil impact in IBD pathogenesis reveals that eosinophils are closely involved in this process through regulatory activities, interactions with other cells and tissues and effector functions that can often be excessive and damage the host. The impact of eosinophils appears to be especially important in altering the structure and protective functions of the mucosal barrier. The author also tried highlighting an interesting new research direction related to exploring poorly investigated immune responses occurring in the protective mucus layer of the gut. It is, however, apparent that our understanding of IBD pathogenesis, including eosinophil participation in it, remains fragmentary and needs further thorough investigation. For this reason, it would be premature to speculate on possible therapeutic interventions targeting eosinophils in IBD.

Eosinophils in the pathogenesis of CRC

CRC is one of the most frequent oncological conditions with estimated global figures of 1801000 new CRC cases and 861700 deaths due to this disease in 2018[206]. Although there are many good reviews addressing various aspects of CRC pathogenesis[11-15], possible role of eosinophils in this process is usually overlooked despite the existence of reports deserving attention and briefly discussed below.

Eosinophil infiltration is often observed in malignancies, but for different tumours it was reported as either prognostically favourable or unfavourable[3,4,207-210]. Nevertheless, the presence of eosinophils in CRC patients is strongly linked with a decreased disease risk, better prognosis and extended patient survival. Indeed, elevated blood eosinophil counts were associated with a decreased CRC development risk[211] as well as better prognosis[212-214]. Eosinophil infiltration of colorectal tumours is a common phenomenon, and higher numbers of infiltrating eosinophils detected both in the tumour tissue[215-217] and peritumourally[218-220] were repeatedly shown to be prognostically favourable. Despite these seemingly cogent findings, the quoted descriptive clinical studies could not provide any direct evidence of anti-cancer eosinophil action, and possible mechanisms of CRC growth inhibition by eosinophils remain poorly understood.

Tumour-associated inflammation is currently recognised among hallmarks of cancer[11]. Eosinophil accumulation accompanying inflammation-related cancer cell death and proliferation in CRC is one of its components that probably reflects Th2 immune responses enhanced at the expense of Th1 immunity[3]. Besides, eosinophils are likely to stimulate tissue remodelling and tumour-related angiogenesis[3,221]. The latter MBP-modulated effect may in theory promote tumour growth, however only non-cytotoxic MBP concentrations enhanced angiogenesis *in vitro*[221], and significant eosinophil infiltration in CRC is likely to produce high MBP concentrations. Ellyard *et al*[222] argued that some components of Th2-driven inflammation in cancer can be associated with anti-tumour activity of $CD4^+$ Th2 cells collaborating with tumour-infiltrating granulocytes, especially eosinophils that exert regulatory functions. In any case, it is now becoming clear that there are several mechanisms driving eosinophil attraction to tumours and defining their influence on malignant tissue. In particular, it was demonstrated *in vitro* that eosinophil chemotaxis could be induced by necrotic, but not viable cells of neoplastic intestinal epithelium[223]. Experiments in a xenograft mouse model indicated that eosinophil infiltration developed rapidly, involved mostly tumour necrotic areas or capsule regions and was not associated with the presence of $CD4^+$ T cells[224], thus suggesting that eosinophil chemotaxis depended on tumour-derived factors, such as damage-associated molecular pattern molecules (DAMPs) including the nuclear protein high mobility group box 1 (HMGB1)[225]. Notably, the presence of cell-free cytotoxic MBP was confined to the necrotic areas of tumours[224]. There are also reports describing expression of eosinophil attractant ecalectin (variant of galectin-9) by human colorectal carcinoma cell lines[226] and of eotaxin 1 in tumours resected from CRC patients[227]. The latter phenomenon was recently investigated further in a mouse model by Hollande *et al*[228], who found that IL33 expressed by tumour cells induced eotaxin 1 production that led to eosinophil recruitment and degranulation-dependent suppression of tumour growth[228]. Interestingly, eotaxin 1 concentration is negatively regulated by serine protease DPP4, and treatment with DPP4 inhibitor sitagliptin was shown to result in an increase in eotaxin 1 level[228]. Sitagliptin is a drug already approved by the US FDA for hyperglycaemia treatment, and it may potentially be re-purposed as a new anti-tumour agent promoting tumoricidal action of eosinophils[228,229]. Coming back to IL33, its role as an influential modulator of early stages of eosinophil development[22], was

noted in the beginning of this review, but it is also a potent eosinophil activator stimulating their degranulation[230]. Moreover, it was reported that IL33-deficient mice had gut microbiota dysbiosis and were highly susceptible to both colitis and colitis-associated cancer[231], which could also be related to impaired eosinophil-driven responses. Hence, multiple parallel pathways are likely to be involved in generating eosinophil infiltration of colorectal tumours and direct killing of malignant cells, a phenomenon already proven experimentally. In vitro studies by French investigators assessing tumoricidal activity of eosinophils against Colo-205, a human colon carcinoma cell line, have shown that this effect was mediated by eosinophil-produced ECP, TNFα and proteolytic granzyme A[232]. The same group later reported that eosinophil attachment to Colo-205 cells depended on interaction between adhesion molecules LFA-1 and ICAM-1 that was upregulated by IL18[233]. Direct tumoricidal effect of tumour-infiltrating degranulating eosinophils was also observed in model experiments using genetically modified mice[234]. It is, however, obvious that the tumoricidal action of eosinophils *in vivo* may involve multiple interactions with other immune cells. Notably, there is evidence that eosinophils can promote either Th2 or Th1 immune responses, depending on varying cytokine profiles[86,235]. Carretero *et al*[236] have recently shown in experiments with xenograft-bearing mice that tumour-homing eosinophils secreted chemoattractants that guided $CD8^+$ effector T cells to tumours, eventually causing tumour rejection. Thus, it is apparent that different immune response scenarios can be involved in anti-CRC action of eosinophils.

Completing this final section of the review, the author is tempted to briefly mention already discussed ETosis as another possible, but hitherto poorly investigated factor in CRC. One interesting link here is provided by recently published results implicating inflammation-induced ETosis in extracellular matrix remodelling and awakening of dormant cancer cells[237]. As tumour-associated inflammation is a characteristic feature of CRC, and significantly increased extracellular trap formation in these tumours is now proven[238,239], it looks probable that previous reports of CRC-associated increase in the amount of DNA in stool[240,241] or on the surface of colorectal mucosa[190,242] at least partially reflected abundant ETosis occurring within mucus layers contacting tumour surface. In essence, cellular presence in the mucus overlaying colorectal tumour surface is quite similar to that depicted by Figure 3, the abundance of exfoliated malignant cells being the only major difference[190]. Although today there is no published evidence of eosinophil contribution in CRC-related ETosis, this evidence is very likely to emerge soon. In contrast to anti-cancer effects of eosinophils in fully developed tumours, which were discussed above, it is impossible to exclude carcinogenicity of eosinophil-derived DNA traps loaded with highly cytotoxic released granules damaging colonic mucosa. Such a carcinogenic action could be involved at early stages of CRC development, especially in IBD-associated context[243,244]. Conversely, eosinophil-driven ETosis may prevent further tumour expansion at later stages of advanced tumour growth.

The presented analysis of literature establishes a link between eosinophil presence and favourable CRC prognosis, but functional versatility of these multifaceted cells may comprise both anti-cancer and tumour-promoting features. Only several experimental studies addressing eosinophil roles in cancer could be highlighted, however it already becomes transparent that alternative mechanisms involving both direct effector action of eosinophils and complex cooperation with other immune cells, especially T lymphocytes, can be engaged in different circumstances. This fascinating area still poses numerous unanswered questions requiring further intense investigation.

CONCLUSION

Upon the presented analysis of the current literature it can be concluded that eosinophils are now regarded as multifunctional mobile cells routinely involved in controlling and regulating a range of biological pathways and responses in both health and disease. The versatility of eosinophils largely depends on the availability of numerous biologically active substances (cytotoxic cationic proteins, cytokines, growth factors, chemokines, enzymes) stored in their specific granules and ready for rapid release. The *lamina propria* of the human gut is one of the main destinations of eosinophils produced and matured in the bone marrow and transferred through the circulation. In the normal physiological conditions, the most important functions of gut-residing eosinophils appear to be their participation in the maintenance of the protective mucosal barrier and interactions with other immune cells, particularly in providing immunity to microbiota inhabiting the lumen of the gut. In health the latter regulatory role may be more important in the small intestine compared to the colon,

mostly due to structural differences of mucus layers covering epithelial surfaces of these two segments of the intestine. Eosinophils are proven to be closely involved in the development of inflammation in IBD, when their uncontrolled cytotoxic effector functions cause damage to host tissues. However, their roles in CD and UC differ. While eosinophil impact in UC pathogenesis largely corresponds to Th2 immune response pattern, the involvement of these cells in CD pathogenesis is less clear. Eosinophils in IBD appear to be especially important in altering the structure and protective functions of the mucosal barrier and modulating massive neutrophil influx to the *lamina propria* followed by transepithelial migration to colorectal mucus. The author believes that accumulating evidence suggests that IBD-associated inflammatory process expands to the mucus overlaying the internal gut surface, and the presence of eosinophils in this mucus is well documented. It can be hypothesised that colorectal mucus presents a previously unexplored unique milieu for disease-related inadequate immune responses in the gut. These responses involving cytotoxic effects and ETosis exerted by both neutrophils and eosinophils on the both sides of the colonic epithelial barrier act as additional pathogenetic factors leading to colonic epithelium ulceration in IBD. However, further research is needed for testing the proposed hypothesis. Literature analysis also highlights an association between elevated eosinophil levels and better CRC prognosis. Mechanisms behind this link may involve both direct anti-tumour action of eosinophils and complex cooperation of eosinophils with T cells but remain to be elucidated. This challenging area still presents an important goal for future research.

It should be admitted that addressing all interesting topics related to eosinophil role in the gut was impossible for obvious reasons. A huge amount of literature covering gut immunity in health and disease exists, and certain selectivity was inevitable. Nonetheless, the author would like to specifically emphasise his opinion on the importance of investigating colorectal mucus in the context of major colorectal diseases including IBD and CRC.

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REVIEW

Immune suppression in chronic hepatitis B infection associated liver disease: A review

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Abstract

Hepatitis B virus (HBV) infection is one the leading risk factors for chronic hepatitis, liver fibrosis, cirrhosis and hepatocellular cancer (HCC), which are a major global health problem. A large number of clinical studies have shown that chronic HBV persistent infection causes the dysfunction of innate and adaptive immune response involving monocytes/macrophages, dendritic cells, natural killer (NK) cells, T cells. Among these immune cells, cell subsets with suppressive features have been recognized such as myeloid derived suppressive cells(MDSC), NK-reg, T-reg, which represent a critical regulatory system during liver fibrogenesis or tumourigenesis. However, the mechanisms that link HBV-induced immune dysfunction and HBV-related liver diseases are not understood. In this review we summarize the recent studies on innate and adaptive immune cell dysfunction in chronic HBV infection, liver fibrosis, cirrhosis, and HCC, and further discuss the potential mechanism of HBV-induced immunosuppressive cascade in HBV infection and consequences. It is hoped that this article will help ongoing research about the pathogenesis of HBV-related hepatic fibrosis and HBV-related HCC.

Key words: Hepatitis B virus; Hepatocellular carcinoma; Liver fibrosis; Regulatory T cells; Regulatory natural killer cells; Dendritic cells; Monocytes

Core tip: We review that hepatitis B virus (HBV) induces suppressive function of the innate and adaptive immune cells in chronic HBV infection, and highlight that immune suppressive cascade contributes to the mechanism of HBV persistent infection. Further, we analyze the potential effects of HBV-induced immunosuppression in HBV-related fibrosis and hepatocellular carcinoma, thus providing underlying research directions for

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future studies into the pathogenesis of HBV-related disease.

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INTRODUCTION

Despite the presence of vaccines and therapeutic drugs, hepatitis B virus (HBV) infection remains a major global health problem. More than 350 million people worldwide are chronically infected with HBV, and about 1 million people die each year from HBV-related complications[1]. Persistent HBV infection can lead to varying degrees of liver damage, which eventually leads to hepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma (HCC)[2]. HBV belongs to the noncytopathic hepatic DNA virus family which only infect human and orangutan liver cells, and there is no evidence that the HBV can infect other non-hepatocyte cells[3]. HBV infection does not cause direct hepatocyte lesions, and the host's immune response determines whether it clears the virus or induces liver disease. In contrast that HBV-infected adults often develop self-limiting and transient hepatitis, and 95% of infections end with virus removal and the establishment of protective antibodies, the vast majority of neonatal vertical transmission of HBV from mother to child develops into chronic infection[4]. While divergent factors are involved in its pathogenesis, chronic HBV persistent infection is a complex process involving the interaction of the host immune system with the virus, which causes the incapacitation of the innate and adaptive immune response[3]. How HBV regulates the innate and adaptive immune cells, leading to persistent virus infection and further consequences, continues to be a research hotspot. Therefore, in this review, we summarize recent findings regarding the function and impairment of innate and adaptive immune cells, and discuss the potential mechanism of HBV-induced immune suppressive cascade in HBV infection and HBV-related liver diseases.

HBV-INDUCED IMMUNE SUPPRESSION CONTRIBUTES TO PERSISTENT INFECTION

HBV induces immune suppressive monocytes/macrophages

Monocytes/macrophages are important natural immune cells found in peripheral blood and organ tissue, and play multiple roles in the innate and acquired immune responses[5]. Monocytes/macrophage interact with lymphocytes through inhibitory or activating surface molecules. HBV stimulates monocyte/macrophage secretion of transforming growth factor β (TGF-β)[6] and interleukin-10 (IL-10)[7], while inhibiting the secretion of tumor necrosis factor α (TNF-α) and IL-12 induced by toll-like receptor (TLR2)[8,9]. Our study of HBV infection in a humanized mice model found that HBV induces human monocyte/macrophage differentiation into M2 macrophages, expressing IL-10 and other inhibitory cytokines[10]. Recently, we found that the anti-inflammatory cytokines (IL-10 and TGF-β) and inhibitory cell surface molecules [Programmed death ligand 1 (PD-L1) and human leukocyte antigen (HLA)-E] expressed in monocytes in patients with chronic HBV infection were significantly higher than those of healthy control[11]. Further experiments in vitro showed that HBsAg or HBV directly induced the expression of PD-L1 and HLA-E and the secretion of anti-inflammatory cytokines of monocytes from healthy adults[11]. Our group recently reported that HBV induces monocyte production of inflammatory cytokines via TLR2/MyD88/NF-κB signaling and STAT1-Ser727 phosphorylation and inhibits interferon (IFN)-α-induced stat1, stat2, and ch25h expression through the inhibition of STAT1-Tyr701 phosphorylation and in an IL-10-dependent, partially autocrine manner[12]. Therefore, HBV-induced suppressive monocytes/macrophages play a key role in the immune pathogenesis of chronic persistent infection.

HBV induces myeloid derived suppressive cells differentiation

Myeloid derived suppressive cells (MDSCs) are bone marrow-derived cell subsets

with inhibitory functions, which can be divided into two main subgroups as M-MDSC ($CD11b^+HLA\text{-}DR^{low/-}CD14^+CD15^-$) and PMN-MDSC ($CD11b^+$ $CD14^-CD15^+$) according to their phenotypic and morphological characteristics[13]. MDSCs were first discovered in tumor tissue and play a role in the development, metastasis and immune escape of tumors[14]. Recent studies have found that MDSCs play a critical role in chronic HBV infection. The level of peripheral MDSCs in chronic hepatitis B (CHB) patients was significantly higher than that of healthy adults, and the percentage of MDSC cells had a significant correlation with HBV load in the plasma of HBV patients[15] and the mouse model[16]. Moreover, HBV induces monocytes differentiation of into MDSCs through the signal transduction pathway such as ERK/IL-16/STAT3/PI3K, thus inhibiting the activation and function of lymphocytes[17]. Drugs that target MDSCs could restore the responses of HBV-specific T cells from CHB patients *ex vivo* and prevent the increase of viral load in HBV mouse models[18,19]. In vitro, MDSCs secrete arginase and down-regulate the CD3ζ chain by missing arginine, thus inhibiting IFN-γ secretion from HBV-specific T cells[20]. In addition, MDSCs produce suppressive cytokines IL-10 to inhibit T-cell response in CHB patients[21]. MDSC not only directly inhibits T cell response through such mechanisms as arginase but also indirectly influences immunomodulatory function by inducing regulatory T cells (T-reg)[22,23].

HBV impairs the maturation and function of dendritic cells

Dendritic cells (DCs) are the professional antigen presenting cells, which process and present antigen to T cells, and are involved in the production of cytokines that influence T-cell polarization. The studies of DCs subsets in chronic HBV infection have primarily been limited to myeloid DCs (mDCs) and plasmacytoid DCs(pDCs), two populations isolated from the peripheral blood. The frequency of mDCs in CHB patients shows a reduction which could be recovered by antiviral therapy[24]. There is a positive correlation of intrahepatic mDC subsets with serum alanine aminotransferase (ALT) levels and a significant inverse correlation with plasma HBV load[25]. The frequency of $CD80^+$ and $CD86^+$ mDCs showed slight differences between CHB patients and healthy donors after in vitro maturation[26]. It was also reported that PD-L1 expression on mDCs was increased in patients with active hepatitis B[27]. Increased ALT levels correlated with increased PD-L1 expression on mDCs, and impaired IFN-α production by pDCs[28]. Although some studies have reported that the function and frequency of pDCs were analogous between CHB patients and healthy controls[24], it has been demonstrated that HBV infection in pediatric patients showed a decreased frequency of pDCs, and the numbers of pDCs were restored by antiviral therapy[29,30]. The expression of the OX40 ligand was reduced in highly viremic patients while the expression of CD40 and CD86 was elevated in pDCs from CHB patients. Decreased expression of OX40L on TLR9-L-activated pDCs from viremic patients with HBV blocks their ability to induce the cytolytic activity of natural killer (NK) cells[31]. Monocyte-derived DCs (MoDCs) from HBV patients were impaired resulting in a reduction in T cell production of IL-2, TNF-α, and IFN-γ because of lower IL-12 secretion[32]. In vitro, cytokine-induced human MoDCs maturation in the presence of HBsAg or HBV contributed to a significantly more tolerogenic DC phenotype as the reduced release of co-stimulatory molecules and IL-12 production as well as a T-cell stimulatory capacity, as evaluated by IFN-γ production and proliferation of T-cells[33].

HBV impairs NK cell function and induces NK cell differentiation

NK cells are another important innate immune cell, which can effectively and quickly identify and remove virally-infected cells without MHC restriction. NK cells are the major lymphocytes in the liver, accounting for about 30% of liver lymphocytes[34]. In the HBV transgenic mouse model, $CD3^-NK1.1^+$NK cells were found to be the main infiltrating lymphocytes of liver inflammation[35]. Functional defects of NK cells were found in CHB patients, showing a deactivation state[36]. The high level of inhibitory cytokine IL-10 in chronic HBV infection has an obvious inhibitory effect on the production of IFN-γ by NK cells[37]. The function of NK cells can be restored by IL-10 and TGF-β neutralizing antibodies in CHB patients[38].

The immunomodulatory function of NK cells has received much attention in recent years. The IFN-γ secreted by NK cells promotes the function of $CD4^+$ T cells and enhance Th1 polarization[39]. However, under appropriate stimulation conditions, NK cells secrete immunomodulatory factor IL-10[40,41]. $IL\text{-}10^+$ NK cells secrete TGF-β and IL-13, but do not secrete IFN-γ[42]. Our study found that the anti-inflammatory cytokines (IL-10) and inhibitory cell surface molecules (PD-1 and CD94) expressed by NK cells in patients with chronic HBV infection were significantly higher than those of healthy adults. Further, in the co-culture experiment of monocytes and NK cells, HBV-induced suppressive monocytes were found to induce NK cell differentiation into regulatory NK cells (NK-regs) expressing anti-inflammatory cytokines IL-10 by

PD-L1/PD-1 and HLA-E/CD94 conjugates[11]. The regulatory NK cells could not only directly inhibit the antiviral function of NK cells, but also inhibit HBV-specific T cell function by reducing the proliferation of T cells[11]. It has been reported that removing NK cells from peripheral blood can enhance the antiviral function of CD8+T cells[43]. These studies show that HBV-induced suppressive monocytes inhibit HBV-specific T-cell immune response by educating regulatory NK cells, which then leads to a chronic persistent infection of HBV.

HBV induces T cell exhaustion and regulatory T cell differentiation

Early studies have determined that the adaptive immune responses, especially HBV-specific $CD4^+$ and $CD8^+$ T cell immune responses, play a crucial role in virus removal and the immune pathogenesis of hepatitis B[3]. $CD4^+$T cells promote CTL responses and the production of neutralization antibodies, while $CD8^+$ CTLs remove hepatocytes that are infected with HBV. IFN-γ and TNF-α secreted by T cells are critical cytokines that inhibit HBV replication[44,45]. In CHB infection, HBV-specific $CD4^+$ and $CD8^+$T cells did not respond adequately, also known as T cell exhaustion[46], showing a significant increase in the expression of co-inhibitory receptors PD-1, CTLA-4, TIM-3 and CD244 on the surface compared to a substantial decrease in cytotoxicity and cytokine secretion capacity. The long-term exposure to a high concentration of viral antigens is the direct cause of T cell immune tolerance and specific T cell exhaustion. Virus-specific T cells become gradually more exhausted with rising viral load and exhibit weakened effector function[47]. Viral load reduction restores the proportion of T cells and the function of HBV-specific T cells[48].

T-regs are a special subset of $CD4^+$T cells that play a critical role in establishing and maintaining immune tolerance. It was reported that the proportion of peripheral $CD4^+CD25^+Foxp3^+$T-reg cells in CHB patients was higher than that in healthy control and self-clearance of acute HBV infection[49,50], and was positively correlated with serum HBV load[51]. In addition, the proportion of hepatic infiltrating T-reg cells increased in CHB infection[52]. Multiple molecules participate in T-reg mediated immunosuppression, including CTLA-4, IL-10 and TGF-β. For example, IL-10 secreted by HBcAg-specific T-reg inhibited the secretion of IFN-γ from HBV-specific CD4+T cells, and blockade of IL-10 restored the secretion of IFN-γ of HBV-specific CD4+T cells[53]. T-regs from CHB patients could inhibit the proliferation and IFN-γ production of autologous peripheral blood mononuclear cells (PBMC) mediated by HBV antigen stimulation *ex vivo*[54]. Therefore, MDSCs and T-regs secrete a variety of effector molecules, directly or indirectly inhibiting T-cell responses, resulting in a chronic, persistent HBV infection.

Therefore, the innate immune cells (monocytes/macrophages, DCs, NK cells) and adaptive immune cells (CD4+, CD8+T cells) are dampened in chronic HBV infection. As shown in Figure 1, HBV induces immune suppressive cells, such as MDSCs, NK-reg, and T-reg cells, to form an immunosuppressive cascade through inhibitory molecules, such as PD-L1, PD-1, IL-10, which contributes to chronic and persistent HBV infection.

DOES HBV-INDUCED IMMUNE SUPPRESSION CONTRIBUTE TO LIVER FIBROSIS?

Liver fibrosis, which is a major global health problem for the lack of effective treatment, is caused by chronic liver injury of any etiology such as viral infection, alcoholic liver disease, and NASH[55]. The associated signals of liver injury cause the activation of hepatic stellate cells (HSCs), the activated HSCs trans-differentiate into myofibroblasts, and become the main source of extracellular matrix in the liver, leading to liver fibrosis. It is thus generally believed that HSCs are essential in the progression of liver fibrosis[56]. HBV is the leading risk factor for liver cirrhosis and HCC. Liver fibrosis is the early stage of liver cirrhosis and HCC, and can be reversed. Therefore, many studies are focusing on the immunopathogenesis of chronic HBV infection and the related liver fibrosis[57]. There have been few reports about the impact of HBV-induced immune suppression on fibrosis.

HBV-induced suppressive monocyte/macrophage in liver fibrosis

HBV interacts with receptors such as TLR2/4, which are expressed on Kupffer cells to produce a large number of inflammatory cytokines and chemokines (TNF, CCL2) causing liver damage[58,59]. These inflammatory mediators induce peripheral monocytes to infiltrate into the liver, and then proliferate and differentiate into macrophages and exacerbate the production of inflammatory cytokines and chemokines, further inducing the development of liver inflammation and fibrosis[60-62].

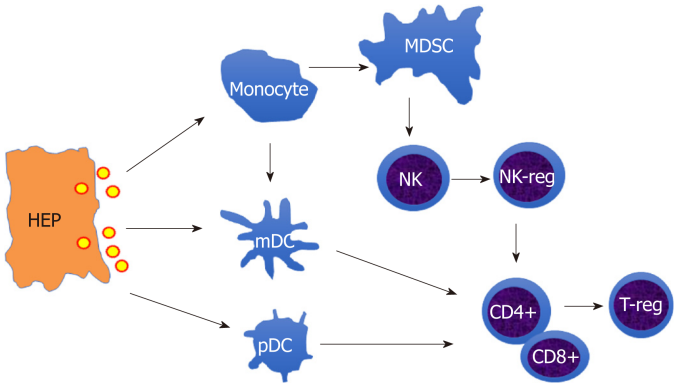

Figure 1 The schematic outline of hepatitis B virus-induced immune suppression network. The infected hepatocytes release hepatitis B virus virion to induce suppressive monocytes (Myeloid derived suppressive cells) and dendritic cells (tolerogenic dendritic cells), which initiate directly inducible T-reg to inhibit T cell activation or mediate indirectly by educating natural killer cell (NK) cells differentiation into NK-reg. HEP: Hepatocytes; MDSC: Myeloid derived suppressive cells; DC: Dendritic cells NK: Natural killer cell; T-reg: Regulatory T cell.

HBV-induced suppressive monocytes/macrophage, on the one hand, produce immunomodulatory molecules (IL-10, TGF-β, PD-L1/2) that inhibit the anti-fibrotic effects of NK cells and T cells; on the other hand, they secrete cytokines such as PDGF and TGF-β to activate HSC, prompting HSC survival[63,64]. A recent report showed that peripheral and ascitic MDSC numbers increase in cirrhosis and HCC, but its role in such pathology was not determined[65]. Conceptually, HBV-induced MDSCs impair the anti-fibrotic function of both T cells and NK cells by inhibiting IFN-γ secretion through PD-L1 or CTLA-4[66]. MDSC also inhibited cytotoxicity of NK cells through PD-L1 or TIGIT[67,68], thus protecting activated HSC from NK cell killing.

HBV-induced suppressive NK cells in liver fibrosis

NK cells secrete IFN-γ that inhibit HSC activation by abrogating profibrogenic TGF-β signaling and induce activated HSC apoptosis[69,70]. In addition, NK cells play a key role in the limitation of liver fibrosis through cytotoxicity of activated HSC[71]. Our recent study found a novel mechanism by which NK cells kill HSCs through TRAIL-involved degranulation manner[72]. In HBV infection, HBV-induced suppressive monocytes induce NK cell differentiation into NK-reg with decreased production of IFN-γ and increased IL-10[11]. Thus, it seems that HBV-induced NK-reg could promote HSC activation. But there is no evidence of the interaction between NK-regs and HSC, although it has been reported that HSCs modulate NK cells through a TGF-β-dependent emperipolesis in chronic HBV infection[73].

HBV-induced T cell immunosuppression in liver fibrosis

IL-2 secreted by CD4+ T cells is important in mediating the anti-fibrotic function of NK cells, and is impaired in HIV/HCV co-infection, which causes rapid progression of liver fibrosis[74]. More likely, HBV-induced T cell immunosuppression may also impair the anti-fibrotic function of NK cell. T-regs directly suppress NK cell degranulation of HSCs through CTLA-4, TGF-β1 and IL-8, and indirectly protect HSCs from NK cell killing by inhibiting MICA/B expressed on HSCs through IL-8 and TGF-β1[75]. In HBV-infected patients undergoing surgery for HCC, hepatic Th17 cells and T-reg were heightened in patients with advanced-stage HBV-related hepatic fibrosis[76]. On the other hand, T-regs could attenuate liver fibrosis by suppressing inflammation[77]. The role of HBV-induced T-regs in fibrosis needs to be further determined.

DOES HBV-INDUCED IMMUNE SUPPRESSION LEAD TO HCC?

HCC is the fifth most prevalent cancer in men and the second principal cause of cancer deaths worldwide[78]. HCC prevalence is very high in China, but the morbidity of HCC has also rising in the United States over the past few decades[79,80]. Early diagnosis and surgical resection are still the key to potential treatment, however, most patients with HCC have advanced-stage tumors with poor prognosis. HBV is one of the major risk factors for HCC, especially in areas where HBV is endemic, such as China[81]. The clinical scope of chronic HBV infection ranges from asymptomatic

carrier status to CHB, which may evolve into liver cirrhosis and liver cancer[82]. It is estimated that 8%-20% untreated CHB adults develop cirrhosis of the liver within 5 years[83], and 2%-8% of those with cirrhosis develop HCC annually[84].

HBV prompts HCC development through direct and indirect mechanisms[85]. Chronic liver inflammation, insertional mutagenesis, and the host gene activation (*cis*' effect) and transactivation by HBx and S proteins and oncogenic co-operativity (*trans*' effect) were proposed as underlying mechanisms of HBV-related HCC development[86-88]. However, the underlying immunopathogenesis of HBV-related HCC development and progression is still not clear. There is a consensus that the immune system is critical in determining the clinical fate of HCC patients[89]. Viruses may also reprogram their immune microenvironment to induce immunosuppression and peripheral tolerance during chronic infections and eventually, tumourigenesis[90]. A meta-analysis of two immunosuppressed populations (transplant and HIV/AIDS patients) revealed a significantly increased incidence of several types of cancer, most of which were pathogen-driven[91]. Immunodeficiency, rather than other risk factors, is responsible for the increased incidence of cancer. The microenvironment of HBV-related HCC is more immunosuppressive than that of non-viral-related HCC[92]. Therefore, HBV-induced immune suppression may play a crucial role in HCC development and progression.

HBV-induced MDSCs in HCC

An increased frequency of $CD14^+HLA\text{-}DR^{-/low}$ MDSCs was reported in both peripheral blood and tumor tissue of HCC patients[93]. Depletion of MDSCs restores production of granzyme B by $CD8^+$ T cells and increases the number of IFN-γ producing $CD4^+$ T cells in HCC patients[66], suggesting that HBV-induced MDSCs inhibit T cell anti-tumor effect. However, the frequency of $CD14^+PD\text{-}L1^+$ MDSCs is only positively correlated with HBV DNA load at the HCC stage[94]. Because of higher levels of $PD\text{-}1^+$ $CD8^+$ T cells in tumor tissues compared to nontumor tissues in HCC[95], PD-L1 expression induced by either HBV or HCC on monocytes/ MDSCs could be associated with impaired T-cell function in HBV-related HCC[66,96] although it has not been demonstrated whether HBV-induced suppressive monocytes were involved in pathogenesis.

HBV-induced NK-reg cells in HCC

NK cells from healthy donor PBMCs have significant cytotoxic function to HCC cell lines, and HepB3 cells transplanted in mice deficient of lymphocytes and NK cells (NOD/scid IL2RGnull) are significantly ostracized by i.p. administered NK cells in an NKG2D-dependent manner[97,98]. It has been reported that PD-1 is highly expressed on peripheral and tumor-infiltrating NK cells from HCC patients, suggesting NK cell exhaustion and poorer survival[99]. Since NK cells play a key role in immunological surveillance, HBV-induced NK cell suppression may play a crucial role in the pathogenesis of HBV-related HCC. As our recent findings[11], PD-L1/PD-1 and CD94/HLA-E signaling control NK cell differentiation to NK-regs which in turn inhibit the anti-viral function of T cells and NK cells. Whether HBV-induced NK-regs are correlated with immune pathogenesis of HCC remains unclear.

HBV-induced T-reg in HCC

Both the absolute numbers and proportion of $CD4^+CD25^+$ T-reg cells significantly increase in the edge region of the tumor, compared to the non-tumor region[100]. In vitro, Huh7 cells inhibit $CD4^+CD25^-$ T-cell proliferation, promote $CD4^+CD25^+$ T-cell proliferation, and enhance their suppressor ability[101]. It seems that the induction of T-regs could be effected by not only the HBV infection but also by the HCC, because the increased $CD4^+CD25^+$ T-regs population and upregulated T-regs-related genes are induced by HepG2.2.15[102]. A decreased infiltration of $CD8^+$ T cells concurrent with abundant accumulation of T-regs was found in tumor regions compared with non-tumor regions[103]. Increased $Foxp3^+$ T-regs not only means poor survival, but also presents a prognostic predictor in patients with early-stage HCC[104]. It indicates that HBV-induced T-regs might be involved in immune pathogenesis of HBV-related HCC.

CONCLUDING REMARKS

Immune suppression induced by HBV infection has been well described by a number of different mechanisms for the different immune cells[10,11,15,27,36,53]. HBV induces immune suppressive cells, such as MDSCs, NK-reg, and T-reg cells, through an immunosuppressive cascade. The excessive immunosuppression could contribute to an HBV persistent infection and the progression of liver fibrosis and HCC[105]. Better

understanding the immunopathogenesis of HBV-related hepatic fibrosis and HCC will be helpful for the intervention and management of HBV progression and the treatment of related end-stage liver diseases in the clinic.

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MINIREVIEWS

Device-assisted enteroscopy: A review of available techniques and upcoming new technologies

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Abstract

The advent of video capsule endoscopy into clinical routine more than 15 years ago led to a substantial change in the diagnostic approach to patients with suspected small bowel diseases, often indicating a deep enteroscopy procedure for diagnostical confirmation or endoscopic treatment. Device assisted enteroscopy was developed in 2001 and for the first time established a practicable, safe and effective method for evaluation of the small bowel. Currently with double-balloon enteroscopy, single-balloon enteroscopy and spiral enteroscopy three different platforms are available in clinical routine. Summarizing, double-balloon enteroscopy seems to offer the deepest insertion depth to the small bowel going hand in hand with the disadvantage of a longer procedural duration. Manual spiral enteroscopy seems to be a faster procedure but without reaching the depth of the DBE in currently available data. Finally, single-balloon enteroscopy seems to be the least complicated procedure to perform. Despite substantial improvements in the field of direct enteroscopy, even nowadays deep endoscopic access to the small bowel with all available methods is still a complex procedure, cumbersome and time-consuming and requires high endoscopic skills. This review will give an overview of the currently available techniques and will further discuss the role of the upcoming new technology of the motorized spiral enteroscopy (PowerSpiral).

Key words: Small bowel disease; Capsule endoscopy; Enteroscopy; PowerSpiral enteroscopy; Endoscopy

Core tip: This review will give an overview of the currently available techniques especially the double balloon-enteroscopy, the single balloon-enteroscopy and the manual spiral enteroscopy. Further the role of the upcoming new technology of the PowerSpiral will be discussed. Available preliminary data on novel PowerSpiral

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Enteroscopy promise a safe and effective tool for deep enteroscopy with a possible faster, deeper and less invasive approach. Further careful evaluation in larger prospective randomized clinical trials is needed to determine the further role of PSE in diagnostic and therapeutic approach to the small bowel.

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INTRODUCTION

Development of endoscopic methods for evaluation of the small bowel started almost simultaneously with flexible colonoscopy. First successful total enteroscopy was reported in 1971 using a ropeway and also a "sonde" method[1]. However, both methods were cumbersome, time-consuming and technically challenging and thus did not achieve wide acceptance in clinical routine. For approximately 30 years, "push"-enteroscopy was the preferred method, leaving the deep portion of the small intestine in-visible and in-accessible to endoscopic evaluation. The advent of video capsule endoscopy (VCE) as a novel non-invasive and reliable method for visualization of the entire mucosal surface of the small bowel in 2000 led to a substantial change in diagnostic assessment of patients with suspected small bowel disorders[2]. The increased detection rate of small bowel diseases consecutively led to an increasing need for a reliable method for direct endoscopic access to the small bowel for histopathological confirmation and/or performance of endoscopic treatment, that is practicable in clinical routine. The development of device-assisted enteroscopy (DAE) in 2001 by Yamamoto[3] established a practical method for examination of the small bowel and resulted in a paradigm shift in diagnostic and therapeutic approach in patients with suspicion of small bowel diseases. Currently three platforms for deep enteroscopy exist: Double-balloon enteroscopy (DBE, Fujifilm, Tokyo, Japan) was first described by Yamamoto in 2001[3], single-balloon enteroscopy (SBE, Olympus Medical Systems Corporation, Tokyo, Japan) in 2007[4] and spiral enteroscopy (SE, Spirus Medical, LCC, West Bridgewater, MA, United States) in 2008[5]. Balloon-guided enteroscopy (BGE, NaviAid, SMART Medical Systems Ltd, Ra'anana, Israel) is not well established in clinical routine, despite a few published trials report a diagnostic yield and DMI not inferior to standard DAE[6,7]. The double-balloon (Fujifilm, Tokyo, Japan)[3] and single-balloon (Olympus Medical Systems Corporation, Tokyo, Japan)[4] enteroscopy systems are the most commonly used devices in Europe. After thorough clinical evaluation SE has gained wide acceptance in North America but less in Europe. Despite these substantial improvements in the field of direct enteroscopy, even nowadays deep endoscopic access to the small bowel with all available methods is still a complex procedure, cumbersome and time-consuming and requires high endoscopic skills. Thus, technique of deep enteroscopy was further developed. In November 2015 clinical evaluation of a novel motorized version of the SE system started with the first in human case of PowerSpiral Enteroscopy (PSE, Olympus Medical Systems Corporation, Tokyo, Japan) being performed by our group[8]. The role of small-bowel capsule endoscopy and DAE for diagnosis and treatment of small bowel disorders was recently addressed in clinical guidelines and technical reviews by the European Society of Gastrointestinal Endoscopy (ESGE)[9,10], American Society of Gastrointestinal Endoscopy[11] and Japanese Gastroenterological Endoscopy Society[12]. This review will give an overview of currently available techniques for deep enteroscopy and will further discuss the role of the upcoming technologies with focus on PSE.

TECHNIQUES

Generally direct endoscopic approach to the small bowel can be achieved from the per-oral route (antegrade) or the per-anal route (retrograde). Enteroscopy has unique challenges due to the length of the small bowel and the difficulties encountered when attempting to push a slim, flexible instrument through as much as 300 cm to 400 cm of

small intestine. Various devices and techniques for enteroscopy have evolved to facilitate endoscope insertion into the small intestine. They are designed to help minimize looping which is the rate limiting step for push enteroscopy. For antegrade approach the endoscope is inserted *via* the mouth passing the esophagus and the stomach before the small bowel can be entered. Insertion depth to the small bowel is usually referenced to the pylorus or the Ligament of Treitz. For the retrograde approach the enteroscope first has to pass the colon before passage of the ileocecal valve facilitates access to the ileum. Non-invasive small bowel imaging modalities, *e.g.*, VCE or magnetic resonance imaging (MRI), are usually performed prior to direct enteroscopy to: First, identify any mucosal or subepithelial lesions indicating direct enteroscopy and thus, improving diagnostic yield of DAE; Second, to decide whether to start with antegrade or retrograde approach, and third, to rule out contra-indications for deep enteroscopy, *e.g.*, severe strictures. DAE with DBE, SBE and conventional SE allows for diagnostic and therapeutic deep enteroscopy and also endoscopic retrograde cholangio-pancreaticography (ERCP) in patients with altered anatomy[13,14]. However, currently available single- and double-balloon enteroscopes with a working length of 200 cm have a working channel of 2.8 mm or less, making the advancement of accessory material sometimes difficult or even impossible[15]. Conventional SE is liable to the same limitations, because the Endo-Ease overtube (Spirus Medical, LCC, West Bridgewater, MA, United States) is usually used with the standard slim 200 cm double- and single-balloon enteroscope[16]. To overcome these limitations, recently new therapeutic enteroscopes for double- and single-balloon platform have been developed with larger working channels of 3.2 mm to reduce friction during introduction of accessory material and facilitate therapeutic interventions[17,18]. Short length of the insertion portion additionally allows for utilization of standard instruments for therapeutic interventions, *e.g.*, sphincterotomes or delivery systems for plastic or self-expandable metal stents.

The choice of the device utilized for DAE mainly depends on the experience and equipment of the endoscopic center and the indication for enteroscopy in the individual patient. In principle, balloon-based techniques, comprising of balloon-assisted enteroscopy (DBE, SBE) and BGE, have to be distinguished from spiral-based technique (SE, PSE). Double-balloon (DBE), single-balloon (SBE) and SE have been studied in numerous uncontrolled and a limited number of controlled trials[19-29]. Advantages and disadvantages of current technologies have been summarized in several reviews and discussed in recent editorials[15,30-36]. In the following technical details of the DAE procedures will be explained. Currently available endoscopes for each technique are listed in Table 1.

Double-balloon enteroscopy (Fujifilm Inc, Tokyo, Japan)

DBE was introduced in 2001 in Japan by Yamamoto as the first method for device assisted enteroscopy[3]. The DBE system combines a flexible endoscope, an overtube and a balloon-pump-system. DBE utilizes a distal and proximal balloon mounted onto the endoscope and overtube tip, respectively, that can be inflated and deflated independently from each other to "anchor" and move the bowel, thereby assisting the operator in advancing the endoscope while gathering the bowel onto the overtube shaft by insertion and retraction ("push-and-pull"-method).

There are three types of DBE available and they include a diagnostic, a therapeutic and a short model (EN-580T, EN-580XP, EI-580BT). The "short" Double Balloon Endoscope is engineered to overcome technically-challenging therapeutic ERCP procedures in patients with surgically-altered anatomy such as Roux-en-Y reconstruction after biliopancreatic, gastric or bariatric surgery.

Single-balloon enteroscopy (Olympus Medical Systems Corporation, Tokyo, Japan)

Beside DBE, SBE is the most popular DAE device used in Europe. In contrast to DBE, SBE has only one balloon at the distal end of the overtube, what simplifies the preparation of the scope prior to start the procedure[4]. On the other hand technique for anchoring the endoscope's tip differs from DBE, because SBE uses scope tip angulation and suction instead of balloon inflation to maintain a stable position ("hook-and-suck"-technique) while advancing the overtube. One diagnostic and one therapeutic model of endoscope are available (SIF-Q180 and SIF-H290S).

Balloon-guided endoscopy (NaviAid, SMART Medical Systems Ltd, Ra'anana, Israel)

BGE utilizes a dedicated through-the-scope balloon which is inserted in the working channel of the endoscope. The balloon aids to anchor a standard endoscope, *e.g.*, colonoscope, in the small-bowel. Progression is achieved by repeated push-and-pull maneuvers. In the resent published studies the BGE is used form the antegrade and retrograde route. For therapeutic maneuvers the balloon catheter can be extracted. If

Table 1 Currently available device-assisted endoscopes: Technical characteristics

DAE System type	Single-balloon enteroscopy	Short-single balloon	Double-balloon enteroscopy	Double-balloon enteroscopy	Short-double balloon	Balloon-guided enteroscopy	Spiral enteroscopy	PowerSpiral enteroscopy
Company	Olympus Tokyo, Japan	Olympus Tokyo, Japan	Fujifilm Corporation Tokyo, Japan	Fujifilm Corporation Tokyo, Japan	Fujifilm Corporation Tokyo, Japan	Smart Medical Systems Raanana, Israel	Spirus Medical, Stoughton, Massachusetts, United States	Olympus Tokyo, Japan
Endoscope model	SIF-Q 180	SIF-H290S	EN-580T	EN-580XP	EI-580BT	No specific scope	No specific scope	PSF-1
Outer diameter distal end of endoscope	9.2 mm	9.2 mm	9.4 mm	7.5 mm	9.4 mm			11.2 mm
Instrument channel inner diameter	2.8 mm	3.2 mm	3.2 mm	2.2 mm	3.2 mm			3.2 mm
Outer diameter of overtube	13.2 mm	13.2 mm	13.2 mm	11.6 mm	13.2 mm		14.5 mm	18.1 mm 31.1 mm (with spiral)
Total length	2345 mm	1830 mm	2300 mm	2300 mm	1850 mm			2015 mm
Working length	2000 mm	1520 mm	2000 mm	2000 mm	1560 mm			1680 mm
Image Enhancement	NBI (Narrow band imaging)	NBI	FICE (Flexible spectral imaging color enhancement)	FICE	FICE	Depend on endoscope used	Depend on endoscope used	NBI

NBI: Narrow band imaging; FICE: Flexible spectral imaging color enhancement.

necessary, it can be reinserted for ongoing the procedure. BGE is also used as an "on-demand" enteroscopy system, as it can be added to every standard endoscope if needed[6,7,37].

Spiral enteroscopy (Spirus Medical, LCC, West Bridgewater, Massachusetts, United States)

Spiral assisted endoscopy is based on a completely different concept of advancing an endoscope by pleating of the bowel on the instrumentation shaft by active rotation instead of applying pushing force. Principle of SE is the conversion of rotational energy of the spiral into linear force to pull the intestine on the enteroscope[16]. This technique has been widely used for anterograde enteroscopy[20,21,24,26,28]. For this purpose the manually rotatable Endo-Ease Overtube (Spirus Medical, LCC, West Bridgewater, MA, United States) is used with a standard thin flexible enteroscope. The distal end of this dedicated overtube harbors a flexible spiral thread for pleating the small intestine over the overtube. By manually rotating the overtube, the spiral engages the small bowel which is thus pleated onto or unpleated from the overtube, respectively, depending on the direction of the spiral rotation. Spiral assisted endoscopy has been also approved and evaluated for retrograde enteroscopy *via* the anal route[24]. However, use of the Endo-Ease Overtube requires assistance by a second endoscopist for its appropriate use.

Upcoming Novel Technology: PowerSpiral Enteroscopy (Olympus Medical Systems corporation, Tokyo, Japan)

A novel motorized spiral endoscope (Olympus Medical Systems Corporation, Tokyo, Japan) has been introduced into clinical evaluation in November 2015[8]. The PSE consists of a 168 cm long flexible endoscope and is fully compatible with the latest EXERA III endoscopy system (Olympus Medical Systems Corporation, Tokyo, Japan). It is similar to other currently marketed endoscopes in that it incorporates a flexible insertion tube, 4-way deflection capabilities, high-definition imaging, optical image enhancement technology capabilities (narrow band imaging), a large caliber accessory channel of 3.2 mm and a separate dedicated irrigation channel. The system is unique in that it incorporates a user-controlled integrated electric motor embedded in the endoscope's handle to rotate a short flexible, disposable spiral overtube, that is attached to a rotation coupler located on the endoscope's insertion tube. Clockwise and counterclockwise rotation is activated by a foot pedal switch. Motorized, active

rotation of this spiral overtube pleats the bowel on to the endoscope's insertion tube. The system measures and feedbacks the resistance that the spiral rotation applies to the tissue *via* a LED display in order to prevent damage to the bowel[38]. PSE is currently been evaluated for its efficacy and safety in two prospective clinical trials in Europe. Preliminary data is currently only available in abstract form[39]. These show, that PSE seems to be safe and effective for deep enteroscopy. Diagnostic yield of antegrade PSE seems at least equal to standard DAE techniques while PSE seems to offer a faster and deeper approach to the small bowel.

DISCUSSION

In the clinical practice there are three well established device assisted enteroscopy platforms: DBE, SBE and the SE[4,5,40]. There is a couple of uncontrolled and only a limited number of controlled trials comparing the different DAE techniques[10,15,19-36]. The comparison of these techniques is difficult in particular due to differences in selection criteria for indications and study endpoints among the available trials.

Depth of maximum insertion (DMI) is used as an indicator of the capability of each device for deep access to the small bowel and to compare the different techniques. On closer inspection of the DMI there are several limitations of an exact measurement, and thus, leading to only an estimation of the covered distance in most trials[31]. An ESGE technical review of 2018 reports, that DBE seems to be associated with a higher DMI, however, the diagnostic yield as well as the safety profile of DBE, SBE and SE seem to be comparable. ESGE concludes, that these techniques appear equivalent for routine clinical practice[10]. A systematic review by Baniya *et al*[15] of 8 studies including 615 procedures found no significant difference between balloon-assisted enteroscopy and conventional SE in terms of DMI, diagnostic and therapeutic yield as well as AE rate, despite a significant shorter procedure time for SE. Another prospective randomized controlled trial by Moran *et al*[35] showed no significant differences in DMI, diagnostic yield, procedure time and adverse events (AEs) comparing antegrade SBE with SE. In this trial the medium DMI varied from 330 cm for SE comparing to 285 cm for SBE beyond the pylorus. Concerning the DMI and the total enteroscopy rate (TER) the most of the published trials showed a benefit for the DBE comparing to SBE and SE. In contrast a systematic review of 68 trials and two meta-analyses of only randomized controlled studies reported on similar results for depth of insertion, diagnostic and therapeutic yield and complications[29,41,42]. Two back-to-back trials compared manual SE with anterograde DBE. Summarizing, DBE seems to achieve a deeper insertion to the small bowel compared to SE[24,43]. Despite of all benefits of the DBE on the other hand, many trials show a longer procedure time in relation to SBE and SE[9,10,22,24,26,29,33,35,43,44].

On closer consideration to the TER several trials compare the various DAE techniques. A 2011 published systematic review of 23 studies including 1143 procedures showed a TER of only 1% for antegrade DBE. Nevertheless in 44% a total visualization of the entire small-bowel was subsequently possible by adding the retrograde approach[44]. A meta-analysis of 2015 compared four randomized clinical trials (RCTs) and confirmed that DBE had a higher TER than the SBE[29]. In keeping with this, in comparison to SE, DBE showed a significantly higher rate for total enteroscopies in a prospective RCT[26].

DAE generally is considered to be a very safe procedure with an overall AE rate of 0.8% for diagnostic procedures[1]. However, most adverse advents occurred in relationship to therapeutic interventions resulting in higher AE rates of up to 10% in therapeutic situations, mainly comprising of perforations and bleedings[10,44,45-48]. Xin *et al*[44] showed in a systematic review of 12823 procedures of DBE a minor complication rate of 9.1%. The rate of major complications were 0.72%. That included perforation (0.24%), pancreatitis (0.2%), bleeding (0.07%) and other (0.21%)[44]. Comparing DBE and SE, Despott *et al*[49] reported in a multicenter DBE registry a major complication rate of 0.8% in 950 procedures. The German DBE register offered a higher rate of major complications of 1.2% in 3894 cases[46]. Maybe a higher inclusion-rate of therapeutic procedures in this trial was the reason for a higher AE rate. Acute pancreatitis occurred in 9 patients. In all of these patients the DBE was performed by the per-oral route. Regarding conventional SE Akerman *et al*[5,16,32,50] reported a major complication rate of 0.3%. In 2950 patients there were 8 perforations but on the other hand no incidence of an acute pancreatitis[50]. The data allows the assumption, that SE has a lower risk of acute pancreatitis than DBE and SBE.

Summarizing, DBE seems to offer the deepest insertion depth to the small bowel going hand in hand with the disadvantage of a longer procedural duration. Manual SE seems to be a faster procedure but without reaching the depth of the DBE in

currently available data. Finally, SBE seems to be the least complicated procedure to perform. The novel PSE may promise a solution for the dilemma and help to overcome the limitations of currently available DAE techniques, as it seems to have adopted lessons learned from the development of DAE systems. In a first prospective bi-centric trial on antegrade PSE aiming for diagnostic yield of PSE 140 procedures were performed in 132 patients without prior abdominal surgery with suspected small bowel disease. Diagnostic yield was shown not to be inferior to standard DAE. Secondary endpoints of the trial promise a potential for deeper and faster approach. Motorization of the spiral enteroscope seems to simplify the procedure of SE while maintaining the beneficial features of SE promising an even further reduction of procedural duration and providing deeper access to the small bowel. Data on efficacy for total enteroscopy and retrograde approach will be available soon. However, data on PSE in patients after abdominal surgery and with altered anatomy as well as for enteroscopy-assisted biliopancreatic interventions are lacking. An international prospective multicenter trial will soon start enrolling patients to answer these questions.

CONCLUSION

DAE complements non-invasive small bowel imaging technologies like VCE and MRI and offers safe and effective deep direct endoscopic access to the small bowel for diagnostic evaluation and therapeutic interventions. However, available standard techniques are still time consuming and cumbersome to use. Available preliminary data on novel PSE promise a safe and effective tool for deep enteroscopy with a possible faster, deeper and less invasive approach. Further careful evaluation in larger prospective randomized clinical trials is needed to determine the further role of PSE in diagnostic and therapeutic approach to the small bowel.

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MINIREVIEWS

Identifying high-risk individuals for gastric cancer surveillance from western and eastern perspectives: Lessons to learn and possibility to develop an integrated approach for daily practice

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Abstract

Current evidence shows that individuals with gastric dysplasia, severe and extensive gastric atrophy, extensive gastric intestinal metaplasia and the incomplete subtype of intestinal metaplasia are at high risk for gastric cancer (GC) development. There are several approaches to identifying these subjects, including noninvasive methods, esophagogastroduodenoscopy and histology. The main approach in Western countries is histology-based while that in Eastern countries with a high prevalence of GC is endoscopy-based. Regarding asymptomatic individuals, the key issues in selecting applicable approaches are the ability to reduce GC mortality and the cost-effectiveness of the approach. At present, population-based screening programs have only been applied in a few Asian countries with a high risk of GC. Pre-endoscopic risk assessment based on demographic and clinical features, such as ethnicity, age, gender, smoking and *Helicobacter pylori* status, is helpful for identifying subjects with high pre-test probability for a possibly cost-effective approach, especially in intermediate- and low-risk countries. Regarding symptomatic patients with indications for esophagogastroduodenoscopy, the importance of opportunistic screening should be emphasized. The combination of endoscopic and histological approaches should always be considered as endoscopy provides a real-time assessment of the patient's risk level. In addition, imaging enhanced endoscopy (IEE) has been shown to facilitate targeted biopsies resulting in better correlation between endoscopic and histological findings. Currently, the use of IEE is recommended for endoscopic examinations, and the Operative Link for Gastric Intestinal

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Metaplasia or Operative Link on Gastritis Assessment grading systems are recommended for histological examinations whenever available. However, resource limitations are an important barrier in many regions worldwide. Thus, for an approach to be applicable in real-life practice, it should be not only evidence-based but also resource-sensitive. In this review, we discuss the current understanding and approaches to identifying high-risk individuals from western and eastern perspectives, as well as the possibility of an integrated, resource-sensitive approach.

Key words: Gastric cancer; Precancerous gastric lesions; Dysplasia; Gastric atrophy; Chronic atrophic gastritis; Intestinal metaplasia; Dysplasia; Surveillance; Screening; Cost-effective

Core tip: Current evidence shows that patients with gastric dysplasia, severe and extensive gastric atrophy, extensive gastric intestinal metaplasia and the incomplete subtype of intestinal metaplasia are at high risk for gastric cancer development. Key issues in selecting approaches to identifying these subjects are the ability to reduce gastric cancer mortality and cost-effectiveness of the approach. Resource limitations are an important barrier in many regions worldwide. Thus, an applicable approach in real-life practice should be not only evidence-based but also resource-sensitive. In this review, we discuss the current understanding from western and eastern perspectives, and the possibility of an integrated, resource-sensitive approach.

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INTRODUCTION

With approximately 1.0 million new cases diagnosed in 2018, gastric cancer (GC) is currently the 5th most common cancer worldwide[1]. However, it is the third leading cause of cancer-related death as the majority of patients are diagnosed at an advanced stage. The detection of GC at an early stage is crucial, as the 5-year survival rate of GC patients is significantly better when it is managed in the early stages[2]. Furthermore, therapeutic endoscopy techniques, such as endoscopic mucosal resection and endoscopic submucosal dissection, have been successfully applied to cure patients suffering from early GC, thereby allowing the patients to avoid the risks associated with surgery.

Diagnosing the disease at an early stage is challenging, as many GC patients are asymptomatic in the early stages and some patients with advanced-stage disease may have no alarming features[3-5]. As the development of GC is usually preceded by the decades-long progression of a precancerous lesion[6] and the progression of GC from the early to advanced stages takes an average of 44 mo[7], it is important to identify high-risk individuals and to offer them a proper surveillance program.

Current evidence shows that individuals with gastric dysplasia, high-stage gastritis [according to the Operative Link on Gastritis Assessment (OLGA) or Operative Link for Gastric Intestinal Metaplasia (OLGIM)], severe endoscopic gastric atrophy (EGA), extensive gastric intestinal metaplasia (GIM) and the incomplete subtype of GIM are at increased risk for GC development[8-12]. In addition, it is reported that *Helicobacter pylori* (*H. pylori*) eradication does not reduce the GC risk of these individuals[13]. Thus, individuals with these conditions are at high risk for developing GC and should be identified and offered proper surveillance.

There are several approaches to identifying high-risk individuals, including noninvasive methods, esophagogastroduodenoscopy (EGD) and histology. The main approach in western countries is histology-based while that in Eastern countries with high prevalence of GC is endoscopy-based. One important issue that affects these

approaches is cost-effectiveness. Another important and challenging issue, which has not received much attention in current literature, is local resources, as there are some regions within which the risk of GC is high but resources are limited (Figure 1). In this review, we discuss the current understanding and approaches from western and eastern perspectives and the possibility of implementing an integrated, resource-sensitive approach.

IDENTIFYING HIGH-RISK INDIVIDUALS FOR GASTRIC CANCER DEVELOPMENT

There are several approaches to identify subjects at high risk for GC development, including noninvasive methods, EGD and histology. A histological examination is traditionally required for the diagnosis of precancerous gastric lesions. However, endoscopy, especially with modern endoscopic technologies, and biomarkers have been reported to have acceptable accuracy in the diagnosis of precancerous gastric lesions. Currently, the main approach in Western countries is histology-based while that in Eastern countries with a high prevalence of GC is endoscopy-based.

Histological approach

Gastric atrophy and the risk of GC development: One of the first systematic reviews to describe the risk of GC in patients with histologically diagnosed gastric atrophy was recently published[14]. This study, which consisted of 5 studies from Europe and three studies from Asia, found that the annual incidence of GC among patients with gastric atrophy ranged from 0.1% to 0.5%. In addition, it found that patients with gastric atrophy in Asia had a higher risk of GC in comparison to those in Europe.

Atrophy of the gastric mucosa was traditionally defined as the loss of glands[15]. However, agreement among pathologists on the recognition and grading of gastric atrophy has remained elusive when using this definition[16]. Subsequently, it was re-defined as the loss of appropriate glands, which led to a high level of agreement among gastrointestinal pathologists trained in different cultural contexts[17]. In addition, although the updated Sydney system of gastritis classification has been accepted worldwide, it lacks prognostic information. Consequently, the OLGA gastritis staging system, which is based on the understanding that the risk levels of GC are directly related to the extent and severity of gastric atrophy, was proposed[18]. This atrophy-based staging system combines the antral and oxyntic mucosal atrophy scores using the updated Sydney system visual analog scales with the aim of offering clinicians information about the risk of GC[19]. A multi-center study has been conducted to test the correlation between the stages of gastritis, classified according to this staging system, with the risk of GC in different populations[20]. This study showed that the OLGA stage of gastritis mirrored the incidence of GC in populations with different levels of GC risk. Observational studies from populations with different levels of GC risk also consistently showed that neoplastic gastric lesions clustered in high stages (*i.e.*, stage III and IV) OLGA gastritis, supporting the potentially useful application of the system in clinical practice[21,22]. In a prospective cross-sectional study of 439 consecutive dyspeptic outpatients in Italy who underwent endoscopy with standardized biopsy sampling, benign conditions were consistently clustered in OLGA stages 0-II, whereas all neoplastic gastric lesions were clustered in OLGA stages III-IV[21]. In another cross-sectional study to assess the distribution of the OLGA gastritis stages of 280 non-ulcer dyspeptic patients in Vietnam, neoplastic lesions were found to cluster in patients with OLGA stages III-IV as opposed to OLGA stages 0-II[22]. A meta-analysis of 6 case-control studies and 2 cohort studies from Europe and Asia also showed that there was a significant association between OLGA stages III-IV and GC[12]. Recently, two large and long-term follow-up Italian studies confirmed that this staging system reliably predicted the risk of GC development[23,24]. The Kyoto global consensus on *H. pylori* gastritis strongly recommends the use of the OLGA and OLGIM grading systems for GC risk stratification[10].

In summary, the application of the new definition of gastric atrophy as the loss of inappropriate glands has led to a higher agreement in the assessment of gastric atrophy. The risk of GC is significantly associated with the extent and severity of gastric atrophy, and the OLGA staging system has been shown to be correlated with risk of GC in long-term cohort studies.

GIM and the risk of GC development: A nationwide cohort study in the Netherlands reported that the overall annual incidence of GC development in patients with GIM was 0.25% at 5 years[25]. A population-based cohort study in Sweden reported that approximately 1 in 39 patients with GIM who underwent EGD with gastric biopsy for

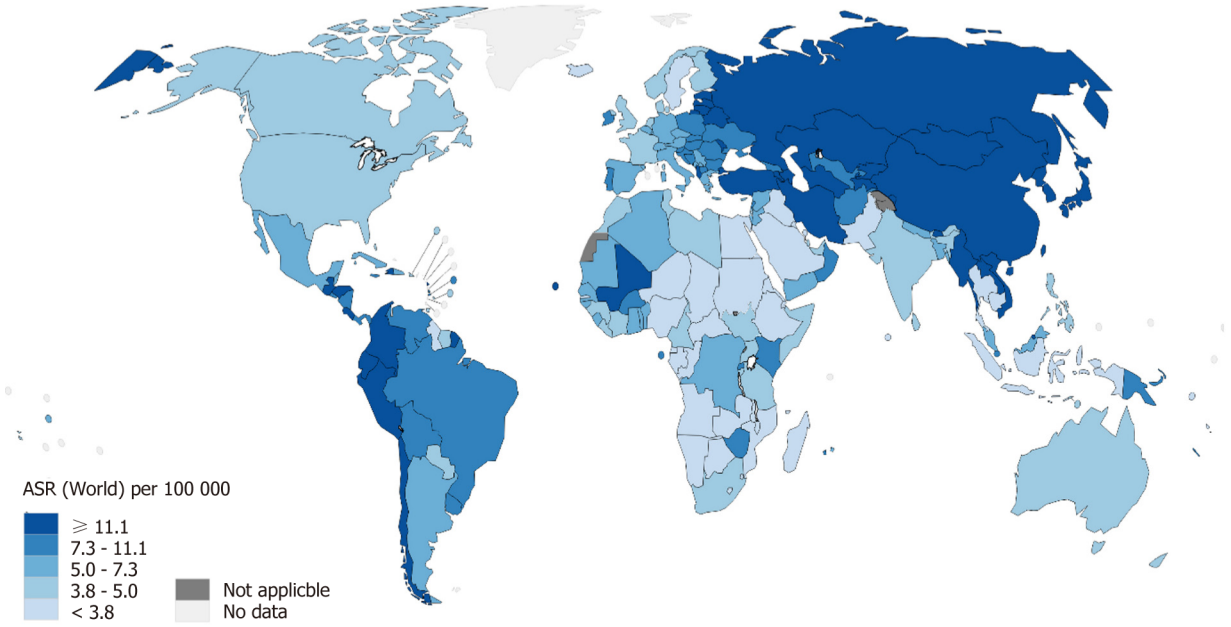

Figure 1 The estimated age-standardized incidence rate for gastric cancer in 2018 (both sexes, all ages)[1].

non-malignant indications developed GC within 20 years[26]. In a recent systematic review consisting of 9 cohorts (4 from the United States, 4 from Western European countries and 1 from South Korea), the incidence of GC among patients with GIM ranged from 0.38 to 17.08 per 1000 person-years[14]. However, the majority of the included cohorts reported incidence rates between 1.26 and 4.10 per 1000 person-years.

GIM subtypes and the risk of GC development: GIM can be classified into complete and incomplete subtypes. The complete subtype (type I) is characterized by goblet cells scattered among columnar absorptive cells. The incomplete subtype is characterized by goblet cells interspersed among mucin-secreting columnar cells, which can be further divided into type II (sialomucin-secreting cells; presence of Paneth cells) and type III (sulphomucin-secreting columnar cells; absence of Paneth cells) by high-iron diamine staining[27].

A cancer registry-based study in Slovenia reported that the cumulative incidence of GC in patients previously diagnosed with GIM was 1.3% in complete GIM type I, 2.8% in incomplete GIM type II and 9.8% in incomplete GIM type III[27]. A Spanish study reported that GC developed in 18.2% of patients with incomplete GIM and in only 0.9% of patients with complete GIM after a mean follow-up period of 12.8 years[28]. This study showed—based on a multivariate analysis—that incomplete GIM was associated with the highest risk of developing GC (Hazard ratio 11.3, 95%CI: 3.8-33.9). In South Korea, GIM subtyping was not found to play a major role in the prediction of GC development[29]. However, these observations were derived from cross-sectional studies and results from follow-up studies are awaited.

In a literature review on the association between incomplete GIM and GC in studies published between 1980 and 2010, 13 of the 14 cross-sectional studies and 6 of the ten follow-up studies found a statistically significant association between incomplete GIM and the risk of GC[30]. Among the studies that reported the magnitude of the risk, the relative risk (RR) of GC in patients with incomplete GIM was 4- to 11-fold higher than that in patients with complete GIM or without incomplete GIM.

In a recent retrospective cohort study in Thailand, 91 patients with GIM were recruited for surveillance EGD every 6-12 mo until a diagnosis of GC was made or the planned 5-year follow-up period was completed[31]. By the end of the study, incomplete GIM and male sex were found to be significantly associated with the development of gastric neoplasia. None of the 81 patients with complete GIM at the time of recruitment developed GC. In contrast, 5 of the 10 patients exhibiting incomplete GIM progressed to high-grade dysplasia (HGD) and GC.

In summary, most of the scientific evidence supports that incomplete GIM is a risk factor for GC.

The extent of GIM and the risk of GC development: The extent of GIM is a very

important risk factor for the development of GC. There are four patterns of GIM distribution[32]. The "focal" GIM pattern consists of scattered foci, mostly in the lesser curvature and incisura. The "antrum-predominant" GIM pattern involves most of the antrum and incisura angularis. The "magenstraße" GIM pattern spreads throughout the lesser curvature from the cardia to the pylorus, also involving the greater curvature of the pre-pyloric antrum. Finally, the "diffuse" GIM pattern involves the entire gastric mucosa, with the exception of the fundic areas. In comparison to the focal or antral-predominant GIM patterns, the magenstraße GIM and diffuse GIM patterns are associated with a 5.7-fold and 12.2-fold increase in the risk of GC development, respectively. An Italian study also reported that the extension of GIM was associated with the risk of GC, and that ≥ 20% baseline GIM extension was a sensitive first screening parameter for identifying subjects with a higher risk of GC[33]. Recently, a Japanese cohort study followed 573 patients for 6.2 years and found that GC developed in 21 patients[34]. The cumulative 5-year incidence of GC was 1.5% in patients without GIM, 5.3% in those with GIM limited to the antrum and 9.8% in those with GIM in the corpus.

The OLGIM staging system: The proposed OLGIM staging system is based on the OLGA staging system, which provides clinically relevant information about GIM[35]. The main parameter of this system is the severity and extent of GIM, rather than gastric atrophy. The rationale for this system is that previous studies reported that the degree of interobserver agreement in the assessment of gastric atrophy was lower in comparison that for the assessment of GIM[36,37].

A prospective multicenter study conducted in the Netherlands found that replacement of gastric atrophy by GIM in the staging of gastritis considerably increased interobserver agreement while the correlation with the severity of gastritis remained at least as strong[35]. A systematic review and meta-analysis on the association between the OLGIM gastritis stage and the GC risk has been recently published[12]. The meta-analysis, which was based on three case-control studies from Eastern countries, showed that the GC risk was significantly higher among patients with OLGIM stage III-IV [Odds ratio (OR) = 3.99; 95%CI: 3.05-5.21: $P < 0.001$]. The only prospective cohort study, which was conducted in the Netherlands, found that patients with OLGIM stage III-IV were more likely to develop HGD (RR = 16.67; 95%CI: 0.8-327.53).

Gastric dysplasia and the risk of GC development: Gastric dysplasia is usually classified as low or high grade[38]. A nationwide cohort study in the Netherlands reported that the annual incidence of GC in patients with LGD and HGD within 5 years after the diagnosis was 0.6% and 6%, respectively[39]. A recent population based cohort study in Sweden reported that 1 in 19 patients with dysplasia progressed to GC within 20 years, although no differentiation was made between those with low-grade dysplasia (LGD) or HGD[26]. Notably, there is a remarkably histological discrepancy between biopsy specimens and material obtained from endoscopic resection. A recent study from Japan found that a substantial proportion of biopsy-proven gastric LGD specimens were diagnosed as GC after endoscopic resection[40]. The strategy for managing patients with gastric dysplasia is, therefore, more straightforward in comparison to that for gastric atrophy and GIM. The resection of endoscopically visible dysplastic lesions is now recommended worldwide, regardless of the grade of dysplasia[9,11,41]. However, a considerable number of patients have endoscopically invisible gastric dysplasia. These patients are still at high risk and need to be strictly followed up[22,42]. A recent literature review reported that LGD persisted in 19% to 50% of patients and that the risk of GC development in these patients ranged from 0% to 23% over 10-48 mo[11]. Regarding endoscopically invisible HGD, immediate endoscopic reassessment with extensive biopsy sampling and surveillance at 6- to 12-mo intervals is mandatory. Furthermore, the disappearance or assumed disappearance of dysplastic lesions, as assessed by follow-up endoscopic biopsy, does not rule out possible progression to invasive GC[9,41].

Endoscopic approaches

White light endoscopy: Gastric atrophy: The endoscopic diagnosis of gastric atrophy based on good visualization of the submucosal vessels, even in the hands of experienced endoscopists, is not reliable[43]. The sensitivity and specificity were only 61.5% and 57.7%, respectively in the antrum; and 46.8% and 76.4%, respectively in the corpus. However, the assessment of EGA according to the Kimura-Takemoto classification has been consistently confirmed to have a good correlation with histological gastric atrophy[22,44]. In addition, several long-term cohort studies confirmed its value in predicting the risk of GC development in subjects with and without *H. pylori* infection, as well as after the successful eradication of *H. pylori*[8,34,45].

The key point in assessing EGA according to the Kimura-Takemoto classification is to identify the location of the so-called endoscopic atrophic border of the stomach[46]. Based on the location of the endoscopic atrophic border, an endoscopic classification of gastric atrophy pattern was proposed which consists of two main types: Closed type (C-type) and open type (O-type). These two types are further subdivided in into three C-types (C-1, C-2 and C-3) and three O-types (O-1, O-2 and O-3). The severity of EGA is often classified into three grades: Mild (C-1, C-2), moderate (C-3, O-1) and severe (O-2, O-3)[8,22,34,45]. Our previous study in Vietnam showed that the severity of EGA was significantly correlated with the OLGA gastritis stage[22]. As EGA assessment has not been widely applied in Western countries and it was unclear whether the EGA findings were correlated with histological atrophy in Western patients, another study was conducted in the United Kingdom[44]. In this study, EGA was compared with histological atrophy using the updated Sydney classification system. The strength of agreement on the extent of atrophy between the endoscopic and histological findings was good, with a weighted kappa value of 0.76. In addition, the strength of agreement between endoscopic and histological atrophy, as assessed by cancer risk-oriented grading (*i.e.*, none, limited atrophy in antrum and angulus or pan-atrophy) was good, with a kappa value of 0.81.

Several studies have consistently confirmed that the severity of EGA at baseline is associated the risk of GC development. A prospective cohort study that followed 1,603 consecutive Japanese patients with benign gastroduodenal diseases for an average of 8 years found that GC only developed in patients with *H. pylori* infection, and the RR of GC in patients with severe EGA at baseline was 4.9 times higher than that of those with no or mild EGA at baseline[8]. A recent cohort study that included 573 Japanese patients who underwent follow-up endoscopy after successful *H. pylori* eradication therapy found that the cumulative 5-year incidence of GC was 0.7%, 1.9%, and 10% in patients with none/mild, moderate, and severe EGA, respectively[34]. Based on the current evidence, the Kyoto global consensus on gastritis suggested that EGA assessment can be used initially in regions with proven expertise in EGA assessment; however, histological confirmation is still recommended[10].

GIM: The endoscopic diagnosis of GIM by standard endoscopy is also unreliable even in the hands of experienced endoscopists. A study in Korea reported that the sensitivity and specificity of endoscopy for the diagnosis of GIM were 24.0% and 91.9%, respectively for the antrum; 24.2% and 88.0%, respectively for the body[47]. A another study in Greece reported that the sensitivity and specificity of endoscopy for the diagnosis of GIM and LGD were 74.6% and 94%, respectively[48].

Gastric dysplasia: The endoscopic diagnosis of gastric dysplasia by endoscopy is even more unreliable, especially for LGD. In a Finnish study that included a series of 101 patients with histologically diagnosed gastric dysplasia graded into three categories (mild, moderate, or severe), all severe dysplastic lesions were detected in visible lesions but 3 (22%) moderate dysplastic lesions and 57 (68%) mild dysplastic lesions were endoscopically invisible and were only detected in random biopsy specimens[42]. Our previous study of 280 dyspeptic patients in Vietnam identified LGD in 7 (2.5%) patients and all of these dysplastic lesions were endoscopically invisible[22].

IEE and magnifying endoscopy: Several studies have shown the significantly higher accuracy of IEE and magnification endoscopy in the diagnosis of gastric atrophy, GIM, dysplasia and GC in comparison to white light endoscopy (WLE). A multicenter prospective randomized study in the Asia-Pacific region was conducted to compare narrow-band imaging (NBI) and high-definition WLE (HD-WLE) in the detection of GIM[49]. This study found that a significantly higher proportion of patients with GIM was detected by NBI compared with HD-WLE (17.7% *vs* 7.7%, $P < 0.001$). Similarly, a recent prospective blinded trial in the United States reported higher proportions of patients with GIM were detected by NBI (65%) and mapping (76%) *vs* HD-WLE (29%) ($P < 0.005$ for both comparisons). In addition, there were also higher proportions of sites with GIM detected with NBI (53%) and mapping biopsies (67%) than HD-WLE (28%) ($P < 0.005$ for both comparisons). A recent consensus developed by expert endoscopists in Asia strongly recommends to use IEE in addition to WLE to improve the detection rate of precancerous gastric lesions[50].

The development of NBI magnifying endoscopy (NBI-ME) has helped endoscopists to better observe the gastric mucosa endoscopically. The Light Blue Crest (LBC) sign, defined as a fine, blue-white line on the crests of the epithelial surface/gyri, was found to correlate with histological evidence of GIM[51]. A recent meta-analysis on the diagnostic yield of LBC in GIM reported that the sensitivity and specificity values of this finding were 0.90 (95%CI: 0.86-0.92) and 0.90 (95%CI: 0.86-0.93), respectively[52]. The practicality of NBI-ME for gastritis staging has been reported in Japan[53]. In this study, the NBI-ME score classification was established from images obtained beforehand, and then biopsy specimens taken from the observed areas were scored

according to histological findings. The NBI-ME and histological stages were assessed using a combination of scores for the antrum and corpus, and were divided into low-risk and high-risk groups. This study found that the agreement between NBI-ME and histological scores was 69.1% for the antrum and 72.7% for the corpus, and that between the high- and low-risk groups was 89.1%. NBI-ME procedures, however, are generally time-consuming and require appropriate training and gastroscope. Therefore, it could not be widely used in daily practice.

A simplified classification system using NBI without magnification has been proposed and validated in Western countries, and its accuracy and reliability in the diagnosis of GIM and dysplasia have been demonstrated[54]. According to the simplified classification, pattern A (regular vessels with circular mucosa) was associated with normal histology (accuracy 83%; 95%CI: 75%-90%), pattern B (tubulo-villous mucosa) was associated with GIM (accuracy 84%; 95%CI: 77%-91%); and pattern C (irregular vessels and mucosa) was associated with dysplasia (accuracy 95%; 95%CI: 90%-99%). The reproducibility of these patterns was high (k = 0.62). Non-experienced endoscopists showed lower agreement (k = 0.6 *vs* k = 0.75) and accuracy (74 % *vs* 86%) than experienced endoscopists, suggesting that appropriate training is required. The real-time validity of HD-WLE with and without NBI in the diagnosis of precancerous gastric lesions and the possibility of deriving a classification for the endoscopic grading of GIM (EGGIM), a score (0-10) resulting from the sum of endoscopic assessments of GIM, have been reported[55,56]. In a later multicenter prospective study, NBI based on the simplified classification was found to significantly increase sensitivity in the diagnosis of GIM and gastric dysplasia (87% *vs* 53% and 92% *vs* 74%, respectively). The area under the curve (AUC) of the receiver operating characteristic curve for EGGIM in the diagnosis of extensive GIM was 0.98[55]. Another study was conducted by the same group to externally validate the EGGIM classification[56]. Consecutive patients underwent HD-WLE followed by NBI to estimate the EGGIM classification. The score was 0, 1, or 2 for no GIM, ≤ 30%, or > 30% of the mucosa, respectively, in five areas (lesser and greater curvature of both the antrum and corpus, and incisura). If GIM was endoscopically suspected, targeted biopsies were performed. If GIM was not noticeable, random biopsies were performed according to the Sydney system to estimate the OLGIM stage. For the diagnosis of high-stage OLGIM gastritis, the AUC was 0.96 (95%CI: 0.93-0.98) and the sensitivity and specificity using a cutoff of > 4 were 89% and 95%, respectively. This study shows the promise of the endoscopic approach in determining the risk of GC development without the need for biopsies. Further studies in other populations should be performed to validate the results.

Non-invasive approaches

Serum pepsinogen (PG) is the most intensively investigated biomarker for precancerous gastric lesions and GC. Serum PG consists of two distinct types, namely, pepsinogen I (PGI) and pepsinogen II (PGII). PG I is exclusively produced by chief and mucous neck cells in the fundic glands, while PG II is secreted by these cells as well as by cells in the pyloric glands and Brunner's glands. Both of serum PGI and PGII levels initially increase on the progression of gastritis. However, as the fundic gland mucosa is reduced, PGI levels gradually decrease while PGII levels remain fairly constant. Consequently, the serum PG I/II ratio (sPGr) decreases in a stepwise manner which is closely correlated with the progression of atrophic gastritis[57]. The low serum PG I level and sPGr, therefore, reflect the severity of gastric atrophy. The measurement of serum PG I and sPGr alone or in combination with *H. pylori* serum antibody (HpAb) test, and/or Gastrin-17 has been investigated to identify high-risk individuals[58].

Non-invasive approaches in western countries: A study was conducted in 284 dyspeptic patients from 14 European countries to evaluate the role of sPGr as a screening test for moderate-to-severe and multifocal atrophic gastritis[59]. The best cut-off point of sPGr was 5.6, which showed 65.0% sensitivity and 77.9% specificity. Another study, which was one of a few population-based studies in Western countries, was conducted to investigate the serum levels of PGI and sPGr in asymptomatic individuals in northern Portugal, a region with high incidence of GC[60]. The participants, whose ages ranged from 40 to 79 years, were classified into a positive test group (PG I ≤ 70 ng/mL and sPGr ≤ 3) and a negative test group (all others). All participants with a positive test result and a consecutive random sample of participants with negative test results underwent EGD and were followed up in 5 years. In the detection of GC development during the follow-up period, this test showed 67% sensitivity, 47% specificity, a positive predictive value of 2% and a negative predictive value of 99%. Recently, a study investigating the cost-effectiveness of population screening strategies based on biomarkers and endoscopy was

conducted in the United States[61]. This study found that although one-time serum PG testing at 50 years of age could prevent one in four cases of GC among men, it was not of high value in improving the outcomes of GC. However, targeting the high-risk group (*i.e*., male, smokers of > 50 years of age) could be a cost-effective approach for reducing GC-related mortality.

Non-invasive approaches in eastern countries: A meta-analysis was conducted to evaluate the prediction of GC development by sPGr, HpAb tests, and a risk-prediction model based on these two tests[62]. This model categorized patients into four groups: low risk (A: HpAb -, sPGr-), moderate risk (B: HpAb+ and sPGr-), and high risk (C: HpAb+ and sPGr+; D: HpAb-and sPGr+). This study included 9 prospective cohorts from Eastern Asian countries with a total of 33741 asymptomatic participants in GC screening programs. The mean ages of the participants at enrollment ranged from 45 to 57 years, while the mean follow-up ranged from 3.9 to 14 years. This study found that adults with a positive sPGr test had an approximately four-fold higher risk of developing GC than those with a negative test. In addition, the four-risk-group prediction model had the potential to stratify middle-aged presumptively healthy adults according to the risk of GC development.

Another meta-analysis, which consisted of over 30000 individuals across 13 different western and eastern countries, was conducted to assess the accuracy of serum PG testing in the diagnosis of GC and chronic atrophic gastritis (CAG)[63]. This study showed that serum PG testing had 69% sensitivity and 73% specificity in the diagnosis of GC, and 69% sensitivity and 88% specificity in the diagnosis of CAG. However, there were significant variations in serum PG measurement methods and cut-off values of PGI and sPGr among the included studies.

One other meta-analysis was conducted to assess the combination of sPGr, gastrin-17 and HpAb tests in the diagnosis of CAG. As Gastrin-17 is only secreted by the G cells of the antral mucosa, a low serum Gastrin-17 level in combination with a positive HpAb test would indicate the presence of antral CAG; and a combination of sPGr, Gastrin-17 and HpAb tests would help to detect the presence and site of CAG[64]. This meta-analysis included 20 eligible studies with a total of 4241 subjects. The median prevalence of CAG across the included studies was 27%. The test sensitivity, specificity, negative predictive value and positive predictive value for the diagnosis of CAG were 74.7%, 95.6%, 91% and 86%, respectively. However, that only six of the 20 included studies considered moderate-to-severe gastric atrophy as a gold standard might have adversely affected the practical usefulness of the study results.

There may be some exceptions regarding the use of the serum PG test in predicting the development of GC in different populations. Notably, a study from Singapore was conducted to examine whether racial differences in the prevalence of *H. pylori* infection and serum PG level could account for racial differences in the incidence of GC[65]. This study found that Indian subjects had a lower incidence of GC but a significantly higher prevalence of low PG in comparison to Chinese and Malay subjects. The study highlighted the limited usefulness of serum PG testing in the Indian population. In addition, the rPGr level has been recently reported to return to the normal range in Japanese patients after successful *H. pylori* eradication[66]. Thus, test results may be misleading in populations in which a large percentage of participants have undergone *H. pylori* eradication (intended and unintended).

LESSONS FROM WESTERN AND EASTERN PERSPECTIVES AND THE POSSIBILITY OF DEVELOPING AN INTEGRATED RESOURCE-SENSITIVE APPROACH TO IDENTIFY HIGH-RISK INDIVIDUALS

Pre-endoscopic risk assessment

The detection of high-risk precancerous gastric lesions generally requires endoscopy with biopsy. However, there are demographic and clinical features that are helpful for predicting the presence of these lesions, including ethnicity, gender, age, family history of GC, *H. pylori* status and serum PG level[4,50,67,68].

A recent review found that individuals in the United States who were immigrants from high-risk regions (East Asia, Russia, or South America) had a higher risk of GC development in comparison to other Americans[68]. In multi-ethnic Southeast Asia countries, it was reported that some ethnic groups, including Chinese, Batak and Minahasanese, had higher risk in comparison to the other ethnic groups[4]. *H. pylori* infection and having first-degree relatives diagnosed with GC have been reported as important risk factors for GC development worldwide[8,67,68]. In addition, male sex,

smoking and advanced age are also associated with a higher risk of developing GC[61,69,70]. The age threshold may differ depending on the GC risk level in each region: it is approximately 40 years in high-risk regions and approximately 50 years in low-risk regions[67-69]. In subjects with family history of GC, the age threshold is approximately 10 years younger than the age of the first-degree relative at the diagnosis or 50 years of age (whichever is earlier)[68]. The application of the PG test in subjects with clinical high-risk characteristics has been shown to be promising but, as mentioned above, should be locally validated.

In summary, pre-endoscopic risk assessment is possible and it is helpful for selecting subjects with a high pre-test probability for a possibly cost-effective approach, especially in intermediate- and low-risk regions.

Approaches for asymptomatic individuals

The two key issues in selecting applicable approaches for asymptomatic individuals are the ability to reduce GC mortality, especially in high-risk regions, and the cost-effectiveness of the approach, especially in low- and intermediate-risk regions. The possibly applicable approaches based on current evidence worldwide are summarized in Figure 2.

Approaches for asymptomatic individuals in eastern countries: At present, population-based screening programs have only been applied in some Asian countries with a high incidence of GC. In South Korea, the national cancer screening program for GC by EGD or upper gastrointestinal series (UGS) was launched in 1999 as a Medicaid program, but it has since expanded to all subjects of ≥ 40 years of age since 2005[71]. A recent nested case-control study was conducted to assess the effectiveness of this program in reducing GC mortality. The study used data from 16584283 subjects who participated in the screening program since 2002, and found that the subjects who received EGD were less likely to die from GC in comparison to those who received UGS[72]. In Japan, a recent cohort study reported that the survival rate and GC mortality among Japanese patients with screening-detected GC were not significantly different from those with interval GC in the annual endoscopic screening program, which also suggested the benefit of endoscopic screening in reducing GC mortality[73]. The current Japanese guidelines for GC screening recommend the use of EGD or UGS for population-based and opportunistic screenings, but emphasize that the former method is more sensitive than the later[74]. Serum PG and HpAb tests are currently not recommended for population-based screening in Japan due to insufficient evidence on the reduction of GC mortality. In China, there have been no national screening programs for GC but some screening programs have been applied in high-risk regions of the country. Among these programs, the so-called two-step examination (*i.e.*, the sequential sPGr-EGD screening method) has been reported to have reasonable cost-effectiveness and good participant compliance[5]. A recent meta-analysis and systematic review, which included 6 cohort studies and 4 nested case-control studies from high-risk Asian countries (2 from South Korea, 2 from China, and 6 from Japan) with 342013 individuals, has been conducted to investigate how endoscopic screening affected the incidence of GC or GC mortality. This study found that endoscopic screening may reduce GC mortality, regardless of the incidence of GC in the included populations. The subgroup analysis showed significant reductions in GC mortality after endoscopic screening in comparison to no screening[75].

In other Asian countries, there have been no national screening programs. We recently conducted a survey about the management of *H. pylori* and GC across 9 South-East Asian countries[4], and found that most lesions were diagnosed in an advanced stage and that the prognosis of GC patients was very poor. Resource limitations are among the most challenging issues for countries with limited resources but a high prevalence of GC. In Singapore's multi-ethnic population, the risk in Chinese is higher in comparison to Malaysians and Indians. A cost utility analysis was conducted to determine whether endoscopic screening for GC would be cost-effective and to better define the high-risk group[69]. This study found that screening of the high-risk group of Chinese men (age-standardized rate, 25.9/100000) from 50-70 years of age was highly cost-effective.

Approaches for asymptomatic individuals in western countries: Most western countries have low or intermediate GC risk (Figure 3). Thus, cost-effectiveness is the main issue concerning the selection of a suitable approach.

The longest follow-up study using serum PG screening tests for GC detection in the West was conducted in the northern part of Portugal, the area with the highest incidence of GC in Western Europe[76]. This cohort included 5913 individuals, of 40-74 years of age, who were subjected to the PG test (PGI = 70 ng/mL and sPGr ≥ 3). This study found that the PG test was suboptimal as a screening test for GC as its

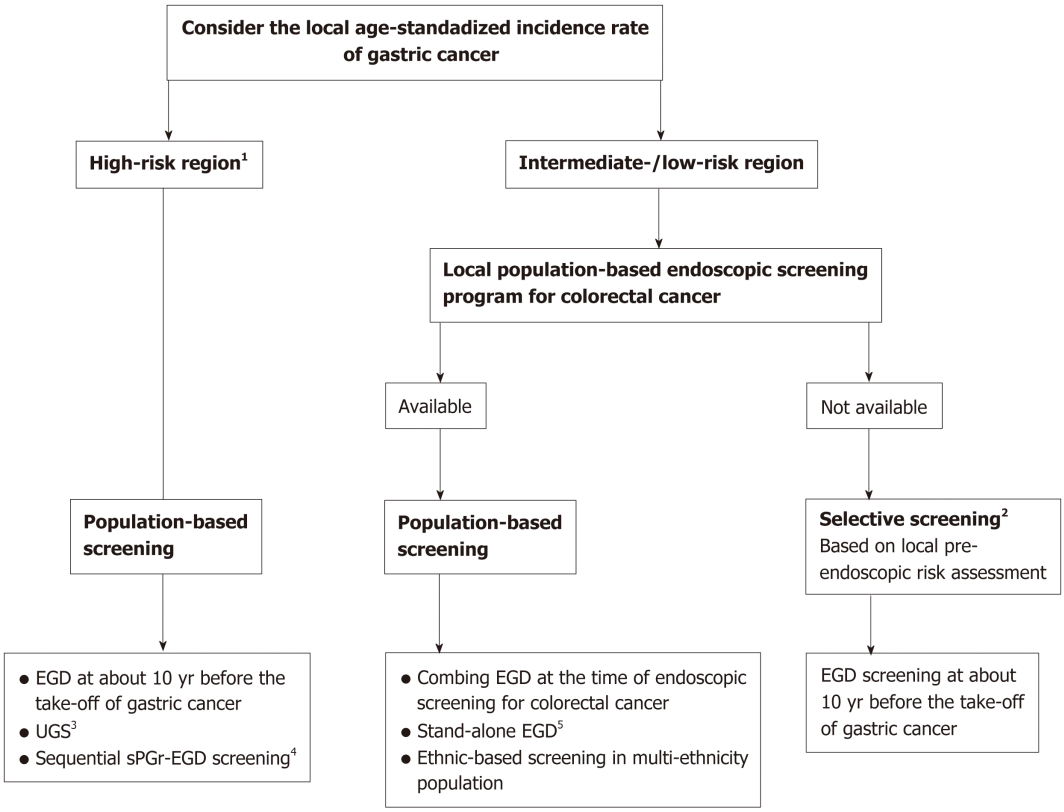

Figure 2 Possibly cost-effective approaches for identifying asymptomatic individuals with a high risk of gastric cancer development. [1]The age-standardized incidence rate is greater than 20 per 100000. [2]Applying selective screening for subjects with high-risk pre-endoscopic features. [3]Not yet shown to reduce gastric cancer mortality. [4]The performance of serum PG I/II ratio should be locally validated. [5]Cost-effectiveness should be considered based on the local age-standardized incidence and the cost of esophagogastroduodenoscopy. In multi-ethnic populations, ethnicity-based screening for high-risk ethnic groups should be considered. EGD: Esophagogastroduodenoscopy; UGS: Upper gastrointestinal series; sPGr: Serum PG I/II ratio.

sensitivity was only 35% at the initial stage and 58% after 3 years of follow-up.

National screening programs for colon cancer have been well-developed in many western countries. Thus, in this region, there have been intensive investigations to determine a reasonable approach for combining gastric and colorectal cancer screening.

An analysis using a Markov model to determine the cost-utility of screening strategies for GC in Portugal was recently reported[77]. The three following screening strategies were compared *vs* no screening: Stand-alone EGD, EGD combined with a colorectal cancer screening colonoscopy after a positive fecal occult blood test or a positive serum PG test. This study found that endoscopic GC screening in Europe could be cost-effective if combined with screening colonoscopy in countries with an age-standardized rate (ASR) of ≥ 10 per 100000. Based on the cost of EGD alone (< €75), the provision of only three EGDs per patient or an ASR > 25/100000 would make stand-alone endoscopic screening cost-effective. Interestingly, this analysis of cost efficacy also supports the national endoscopic screening programs that are currently running in Japan and South Korea (ASR 27.5 and 39.6 per 100.000, respectively)[1].

The marked differences in the prevalence of GC among different ethnic groups in multi-ethnic countries may also affect the cost-effectiveness of the approach. In the United States, a recent study investigated whether selected non-cardia GC screening for members of high-risk ethnic groups was cost-effective[70]. A decision analytic Markov model was developed with the base case of a 50-year-old person of non-Hispanic white, non-Hispanic black, Hispanic, or Asian ethnicity. The cost effectiveness of a no-screening strategy (current standard) for non-cardia GC was compared with that of two endoscopic screening modalities initiated at the time of screening colonoscopy for colorectal cancer: EGD with biopsy examinations and continued surveillance, only if GIM or more severe histological findings were identified, or EGD with biopsy examinations continued every 2 years even in the absence of these histological findings. Compared with biennial and no screening, EGD screening with continued surveillance only when indicated was cost effective for non-Hispanic blacks, Hispanics, and Asians, but not for non-Hispanic whites. The cost-

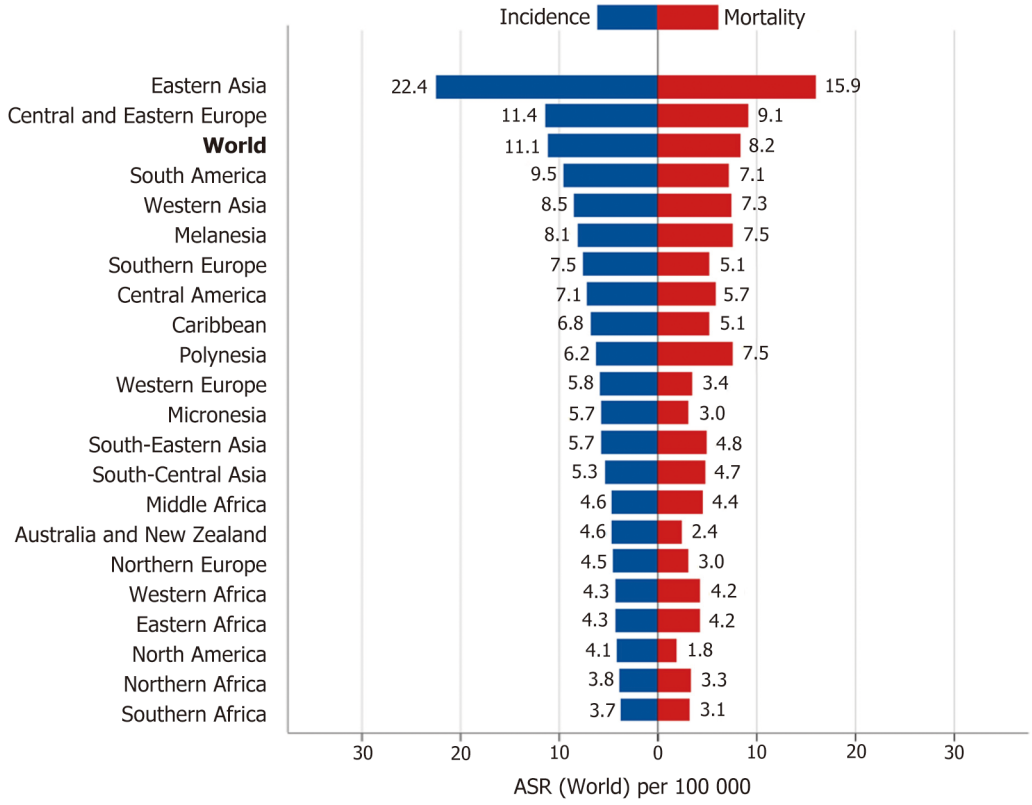

Figure 3 The age-standardized incidence and mortality rates of gastric cancer[1]. ASR: Age-standardized rate[1].

effectiveness was highest for Asians. A selective screening approach based on ethnicity, family history of GC and the age threshold has also been recently proposed in the United States[68].

Approaches for patients who have indications for EGD according to the current guidelines

Definitely, symptomatic patients who have indications for EGD according to the current guidelines for upper gastrointestinal symptoms are the subjects who benefit the most from opportunistic screening, regardless of the GC risk levels in their countries. However, we believe that the importance of opportunistic screening should be further emphasized in guidelines for dyspepsia and gastroesophageal reflux disease management and should always be considered whenever EGD is performed.

The combination of endoscopic and histological approaches should always be considered as it has several advantages. First, endoscopy provides a real-time assessment of the patient's risk of GC development. As endoscopists tend to focus on endoscopic findings that explain patient symptoms, it is important to be cautious that precancerous gastric lesions and even early GCs may already exist. These subtle lesions are often not the causes of the symptom(s) and are very easy to miss[5]. Second, the results of histological examination greatly depend on the location from which the specimens are taken. New endoscopic technologies have helped to improve the endoscopist's ability to identify subtle changes in the gastric mucosa and facilitate targeted biopsies instead of mapping biopsies, which results in a better correlation between endoscopic and histological findings[50,52,55,56].

How to make this combined approach widely applicable in daily practice is a crucial issue. Obviously, an ideal approach should be accurate as well as feasible; it should not be time-consuming or require special expertise or equipment. Currently, the best evidence supports the use of IEE for endoscopy and OLGIM/OLGA grading systems for histological examination whenever applicable. However, resource limitation is an important barrier in many regions worldwide. Notably, there are several countries with a high risk of GC but limited resources, including Mongolia and Vietnam[1]. Resources may also be quite different within the same country, as reported in our recent survey of 9 Southeast Asian countries[78]. Thus, a suitable approach should not only be evidence-based but also resource-sensitive (Figure 4).

Endoscopic strategy: Regions with high resources: Start with WLE and escalate to IEE

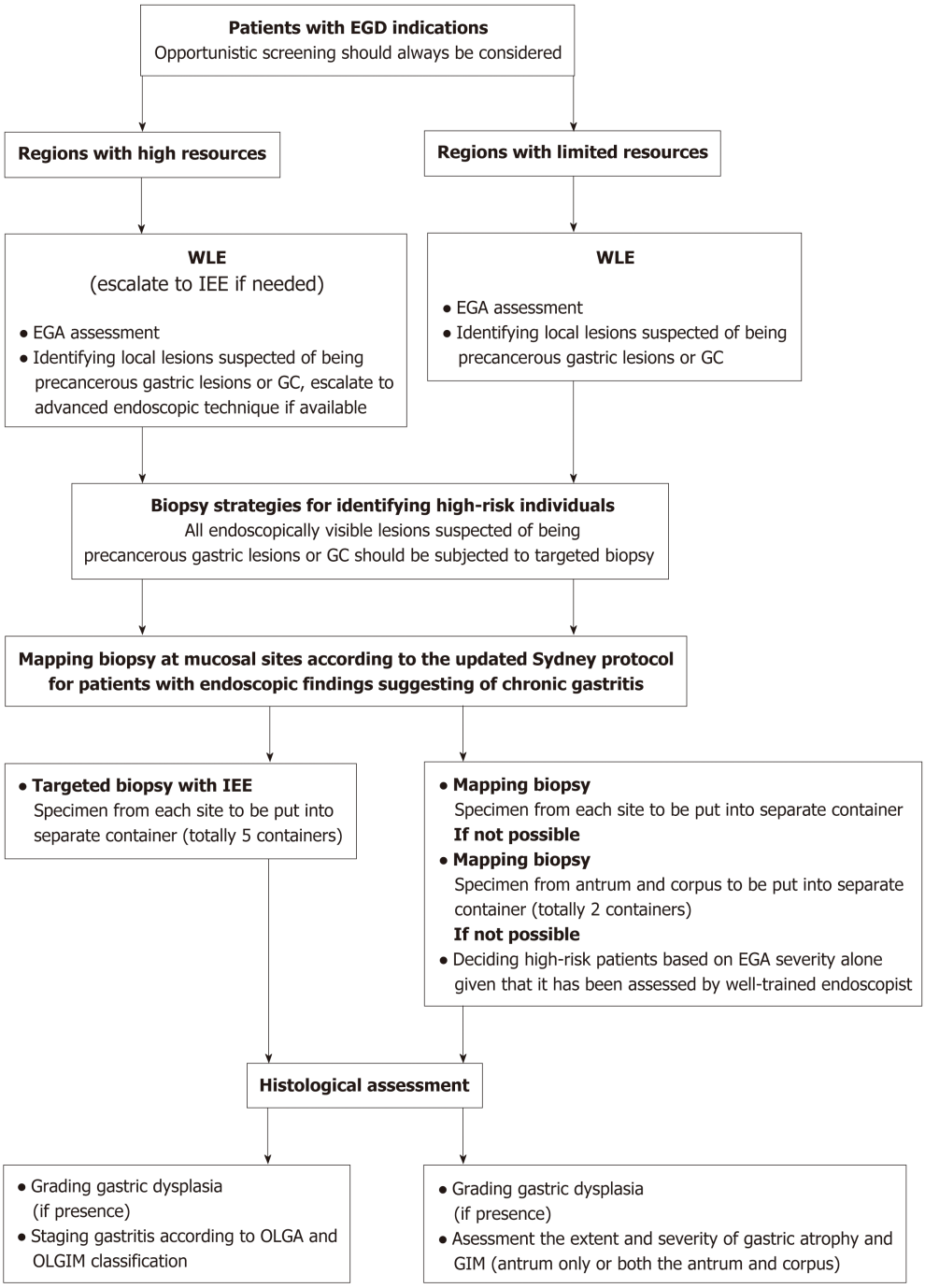

Figure 4 Resource-sensitive approaches to identifying high-risk patients who undergo esophagogastroduodenoscopy for any reason. WLE: White-light endoscopy; EGA: Endoscopic gastric atrophy; GC: Gastric cancer; IEE: Image-enhanced endoscopy.

if necessary. Recent studies from the east and west strongly support that IEE, in addition to WLE, improves the rate at which gastric premalignant mucosal changes such as gastric atrophy and GIM are endoscopically detected[50,55,56].

Regions with limited resources (IEE is unavailable): Start with WLE and evaluate the severity of EGA. Recently, several cohort studies in Asia have shown that EAG assessment can help to effectively identify high-risk individuals with a higher risk of GC development among patients with moderate-to-severe EGA[8,34,45]. A recent global consensus on gastritis recommends that EGA be used if expert endoscopists are available[10]. We previously reported good to excellent intraobserver agreement and a moderate interobserver agreement among experienced endoscopists in the assessment of the severity of EGA[79]. A recent study reported that the interobserver agreement for the diagnosis and grading of EGA significantly improved after proper training and that it remained stable after intervention, irrespective of the endoscopist's experience level[80]. Thus, this endoscopic assessment, which requires no additional equipment, is potentially useful in regions with limited resources. One limitation of EGA

assessment that should be kept in mind is that the improvement of the severity of EGA may not be parallel with histological gastric atrophy after successful *H. pylori* eradication[81]. In such situations, it is necessary to obtain biopsy specimens for histological examination, especially if the baseline histological results are not available.

Biopsy strategy: Obviously, endoscopically visible gastric lesions that are suspected to represent precancerous gastric lesions and GC should be biopsied. However, the strategy of taking mapping biopsies may differ depending on the local resources (Figure 4).

In regions with high resources, the performance of a mapping biopsy at mucosal sites is recommended, according to the Sydney protocol, for patients with endoscopic findings suggestive of chronic gastritis[10]. A biopsy specimen from the angularis angular is essential in order not to downgrade the OLGA and OLGIM gastritis stage and miss high-risk individuals[82]. Specimens from each site should be put into separate container. Whenever available, IEE should be used for detection and obtaining targeted biopsy specimens[50]. Regarding histological assessment, the OLGIM and OLGA staging systems should be applied[9,10]. However, a recent South Korean study found that only about one quarter of GC patients in this high-risk population had high-stage OLGA and OLGIM gastritis. Thus, the sensitivity of these staging systems as indicators for GC in Asians, may be lower in comparison to the sensitivity reported in western populations, and local validation is required[83]. A GIM subtype analysis may be considered but is not a necessity, as the presence of incomplete GIM is significantly associated with extensive GIM, which is a documented marker that is easier to evaluate[32,33,84].

In regions with limited resources, mapping biopsy is also recommended and specimens from each site should be put into separate containers as mentioned above. In some developing countries, the cost of histological examinations is not currently reimbursed and the cost increment of additional containers may not be affordable for many self-paid patients[4]. As patients with extensive gastric atrophy and/or GIM have a higher risk of GC development[32,34,35], a reasonable option is to take 5 biopsies at mucosal sites according to the Sydney protocol and put them into 2 separate containers for the antrum and the corpus. Another option is to define high-risk patients based on EGA severity alone, if well-trained endoscopists are available.

Approach for patients with upper gastrointestinal symptoms who do not yet have indications for EGD according to the current guidelines

The indications for EGD according to the current guidelines on dyspepsia or gastroesophageal reflux disease are mainly to detect organic diseases that cause the patient's symptoms, especially to rule out upper gastrointestinal malignancies. For a long time, the role of EGD as an opportunistic screening tool for these malignancies, including GC, has not been sufficiently emphasized. In our opinion, the benefits from opportunistic screening by EGD should also be considered whenever we decide whether patients with upper gastrointestinal symptoms should undergo EGD. There is currently no evidence on this topic and it represents an important and interesting direction for future research.

CONCLUSION

The characteristics of individuals with high risk for GC development are well recognized. There are several strategies that employ non-invasive, endoscopic, histological or combined approaches in order to attempt to identify these individuals. The main approach in western countries is histology-based while that in eastern countries with high prevalence of GC is endoscopy-based. Although some approaches have demonstrated their cost effectiveness and their ability to reduce GC mortality, these cannot be widely applied in many regions due to resource limitations. Based on the current evidence from both western and eastern perspectives, an integrated and resource-sensitive approach could be developed for real-life practice.

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MINIREVIEWS

Is the treatment outcome of hepatocellular carcinoma inferior in elderly patients?

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Abstract

In view of the increasing life expectancy in different parts of the world, a larger proportion of elderly patients with hepatocellular carcinoma (HCC) requiring oncological treatment is expected. The clinicopathological characteristics of HCC in elderly patients and in younger patients are different. Elderly patients, in general, also have more comorbidities. Evaluation of the efficacy of different HCC treatment options in elderly patients is necessary to optimize treatment outcomes for them. Treatment modalities for HCC include hepatectomy, liver transplantation, radiofrequency ablation, transarterial chemoembolization, and molecular-targeted therapy with sorafenib. In this review, current evidence on the risks and outcomes of the different HCC treatments for elderly patients are discussed. According to data in the literature, elderly patients and younger patients benefited similarly from HCC treatments. More clinical data are needed for the determination of selecting criteria on elderly HCC patients to maximize their chance of getting the most appropriate and effective treatments. As such, further studies evaluating the outcomes of different HCC treatment modalities in elderly patients are warranted.

Key words: Hepatocellular carcinoma; Aged; Clinical outcome; Surgery; Hepatectomy

Core tip: Elderly patients and younger patients benefited similarly from hepatocellular carcinoma (HCC) treatments. Advanced age and comorbidity are intrinsic factors in elderly HCC patients but should not preclude them from receiving treatments. Patients should be evaluated individually and treatment options should be personalized. All treatment options available to the young should be made available to the elderly. Careful assessment of clinical status, cancer stage and comorbidity is needed to ensure good

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common cancer and the third most common cause of cancer-related deaths worldwide[1]. Because of the high prevalence of hepatitis B virus infection[2], countries in eastern and southeast Asia have the highest incidence of HCC in the world[3].

Aging is a major risk factor and poor prognostic factor for most chronic diseases. Owing to the remarkable socioeconomic development and advancement of medical care, average life expectancy has increased around the world; citizens of many developed countries enjoy a lifespan of over 80 years. In the Hong Kong Special Administrative Region, the average life expectancy is the longest in the world. According to a report by Hong Kong Centre for Health Protection, the average life expectancy at birth reached 81.9 and 87.6 years for males and females respectively in 2017. Consequently, an increasing proportion of elderly patients with HCC requiring oncological treatment was expected[4]. It was estimated that the incidence of HCC will increase by approximately 59% by 2030, more than 50% of which will be in people aged 65 or above[5].

HCC in the elderly population may show different clinical and pathological characteristics when compared to the younger population. The incidence rate of HCC raises at the age of forties and decreases after eighties[6]. Aging has been shown to be associated with gradual alteration of hepatic structure and function as well as various changes in liver cells[7,8]. In the sequenced process of liver injury, aging decreases regenerative ability[7,9]. Elderly patients also have inferior cardiopulmonary function. Comorbidities such as diabetes mellitus and renal insufficiency are common among the elderly population. Aging is associated with higher severity and worse prognosis of various liver diseases due to the summation of the aforesaid factors.

Most developed countries accept the chronological age of 65 years as the definition of an elderly person[10]. However, many clinical studies about HCC in the elderly population defined elderly as over 70 years of age[11-17]. This heterogeneity will persist through the discussion in this review. The age cutoff for elderly in the literature varies from 60 to 80, while 70, 75 and 80 were the cutoffs commonly used.

Although the prevalence of hepatitis B virus infection is decreasing in our locality due to the implementation of the universal neonatal vaccination program since 1988, the need for HCC treatment in the elderly may not decrease due to the following three reasons: (1) Delay in HCC recurrence due to improved primary treatment; (2) Increasing incidence of types of liver cirrhosis that require more time to develop into HCC, *e.g.*, non-alcoholic steatohepatitis[18]; (3) Liberal use of antiviral agents which have an effect on viral hepatitis and delay the natural course of HCC formation[19]. In view of the above, evaluation of treatment options for elderly HCC patients is of high clinical relevance. The treatment options of HCC vary according to the stage of the disease. They include hepatectomy, liver transplantation (LT), radiofrequency ablation (RFA), transarterial chemoembolization (TACE), and molecular-targeted therapy with sorafenib. However, whether or not age should be a factor of consideration in HCC treatment allocation is controversial.

The aim of this review is to give an overview of the current knowledge on HCC treatments in elderly patients and to provide available evidence on the treatment modalities for HCC. In particular, the discussion will focus on the role of each treatment in the management of elderly HCC patients and these treatments' respective clinical impact.

HEPATECTOMY

Hepatectomy is an established therapeutic modality for HCC[20]. The mortality rate of liver resection is minimal[21] although the morbidity rate is 20%-30%[22-25]. The surgical

outcome of hepatectomy depends on host liver function, HCC staging, and anesthetic risk[20]. In addition to the fact that young and old patients have histological difference in liver, elderly patients usually have more comorbidities, such as cardiovascular problems, diabetes mellitus, hypertension, and pulmonary disease. As such, they tend to suffer worse surgical outcomes[26].

Numerous studies reported the results of hepatectomy for HCC in elderly patients. However, the majority of them were retrospective observational studies and therefore the validity of these results is debatable[27-36]. There are a few meta-analyses comparing hepatectomy outcomes between the elderly and the young, and Table 1 is a summary of these meta-analyses[37-39]. In general, comparable short-term outcomes (mortality, morbidity, immediate surgical complications, *etc*.) between the elderly and the young were reported. Long-term outcomes (1-, 3- and 5-year overall and disease-free survival rates) were also comparable between the two groups of patients in all the meta-analyses. The results were surprising as it would have been expected that the elderly would have worse post-hepatectomy outcomes due to their impaired physiological functions. In fact, one of the analyzed studies even reported better 1-year survival in the elderly compared to the younger group (odds ratio = 0.762, P = 0.045). This could probably be explained by the more careful selecting criteria on elderly patients – lower risk for surgery and lower grade of background liver fibrosis[40]. Heterogeneity of study design and publication bias might also have led to the paradoxical result.

There are different scoring systems for assessing elderly patients with poor physiological status, including Possum/pPossum, E-PASS, and APACHE II score. Although age is not an accurate risk factor for mortality or morbidity, it usually correlates with physiological reserve and is a surrogate marker for comorbidities[15,41,42]. The proportion of comorbid disease (cardiovascular, cerebrovascular and renal diseases, chronic obstructive pulmonary disease, *etc*.) is higher in elderly patients than in their younger counterparts. Cardiopulmonary workup including echocardiography and lung function test should be performed, if necessary, for patients with a high index of suspicion of occult cardiopulmonary disease[43]. Perioperative patient evaluation by physicians and anesthetists would optimize the outcomes of hepatectomy in elderly patients.

Laparoscopic hepatectomy was introduced in 1996[44] as a minimally invasive technique with potential advantages. Its potential benefits over open hepatectomy include decreased operative blood loss, decreased pain, better cosmesis, faster recovery, fewer cardiovascular and respiratory complications, and shorter hospital stay. Many studies have shown that it could attain better perioperative outcomes[43,45-47]. Although laparoscopic hepatectomy has been shown to be a safe and effective approach to the management of liver disease[48-52], its application to elderly HCC patients with cirrhosis and other comorbidities remains unclarified. For this potentially feasible and safe alternative to open hepatectomy to benefit more patients, selecting criteria on elderly patients need to be determined.

LIVER TRANSPLANTATION

In the past, LT was seldom performed in elderly patients[53]. Since the 1990s, cases of LT in the aged population have gradually been reported and its feasibility in treating the elderly has been evaluated[54-56]. However, most of these studies used a cutoff age of 50-60 years, which would be controversial nowadays in view of the improved life expectancy worldwide. A more recent retrospective study reported a mean post-LT survival of 65 mo in 13 patients who were 75 or older at the time of LT[57].

Data in the literature about LT for elderly HCC patients was very limited since HCC is not the only indication for LT. In the several studies that evaluated the outcomes of LT in elderly patients[58-64], there were inconsistencies in the results: some reported comparable survival outcomes between older and younger recipients while some reported significantly worse survival outcomes in the elderly group. The studies quoted were all retrospective in nature with questionable validity, and the cutoff used for old age also varied in the studies, making direct comparison difficult. However, the researchers in the different studies generally agreed that age should not be the only factor in determining eligibility for LT. It is believed that other patient factors like preoperative disease severity (MELD score) and functional reserve are more important than chronological age in determining eligibility for LT[65]. Since elderly HCC patients often have a lower grade of background liver fibrosis and lower Child-Pugh scores when compared to younger HCC patients[40], LT may potentially be a treatment option for elderly HCC patients. Studies specifically evaluating LT as an oncological treatment for elderly HCC patients need to be conducted before patient

Table 1 Summary table of meta-analyses comparing outcomes of hepatectomy for hepatocellular carcinoma in the elderly and young populations

Authors (year)	Characteristics of included studies	Outcome measures and results	Conclusions
Hung *et al*[38], (2015)	23 studies included in total 18 studies on hepatectomy for hepatocellular carcinoma (6341 patients)	**Short-term outcomes** Treatment complications: Comparable between the elderly and younger groups **Long-term outcomes** 1-, 3-, 5-yr disease-free and overall survival: Increased 1-yr overall survival in the elderly compared to the younger group Comparable 3- and 5-yr survival, disease-free survival	Hepatectomy, transarterial chemoembolization and radiofrequency ablation are safe and effective for elderly hepatocellular carcinoma patients Similar success compared to younger patients Optimal strategy depends on patient and tumor characteristics (evaluation of cancer stage and general condition is important)
Mizuguchi *et al*[37], (2014)	16 studies included in total 5 studies on hepatectomy for hepatocellular carcinoma (1932 patients)	**Short-term outcomes** Morbidity and mortality: No significant differences between the elderly and younger groups	Outcome of hepatectomy depends on tumor type (hepatocellular carcinoma *vs* colorectal metastatic cancer) Hepatectomy is indicated in older hepatocellular carcinoma patients
Zhou *et al*[9], (2013)	28 studies included in total 11 studies on hepatectomy for hepatocellular carcinoma (3560 patients)	**Short-term outcomes** Morbidity and mortality: No significant differences between the elderly and younger groups **Long-term outcomes** 5-yr disease-free and overall survival: No significant differences between the elderly and younger groups	Similar overall morbidity and mortality in elderly and young patients Analysis should be interpreted with caution as elderly mortality after hepatectomy has been reported to be higher in the presence of cirrhosis Age alone should not be a contraindication to hepatectomy

selection criteria can be determined and evidence on outcomes can emerge.

The risk-benefit profile is the most important parameter for consideration of high-risk procedures such as LT for elderly patients. Data on the benefit of LT to elderly HCC patients are lacking, but we can assume that LT, as an oncological treatment, offers the elderly and the young similar benefit, as do other HCC treatment modalities. However, as depicted in Figure 1, the operative risk is considerably elevated in the older population. Therefore, LT as a treatment option for the aged population is still subject to debate, and the upper age limit for undergoing LT is yet to be defined.

In clinical practice, LT is seldom performed on elderly patients. In Hong Kong and other Asian regions, the rates of organ donation from the deceased are low[66]. The utilization of deceased-donor liver grafts for elderly HCC patients would be subjected to evaluation based on treatment efficacy and cost-effectiveness. Donor risk would be an additional concern in the case of living-donor LT. Furthermore, elderly waitlisted patients have a higher dropout rate than their younger counterparts, mainly due to deterioration of cardiopulmonary status.

RADIOFREQUENCY ABLATION

Hepatectomy, LT and RFA are considered curative modalities for early-stage HCC. Percutaneous RFA would be beneficial to elderly patients with a poor risk profile due to avoidance of risks associated with general anesthesia. It is also less invasive and hence has fewer periprocedural risks and deteriorative effects on liver function[67]. It has become an increasingly popular treatment option for elderly HCC patients. AASLD and EASL guidelines present RFA as a potential treatment option for compensated cirrhotic patients with small HCCs < 5 cm[68,69]. However, to date, there have not been many studies about the long-term outcome of RFA in HCC patients. Percutaneous ethanol injection has been used to treat elderly HCC patients but evidence of its efficacy is limited[70,71]. RFA has largely replaced percutaneous ethanol injection for better recurrence-free survival and fewer treatment sessions[72].

In the comparison of RFA and surgical resection by postoperative outcomes, contradictory results were yielded. Peng *et al*[73] reported that patients having RFA had better outcomes than those having surgical resection, while Bauschke *et al*[74] and Yu *et al*[75] reported better outcomes for surgical resection. Another retrospective study by Jiang *et al*[76] concluded that RFA should be recommended for elderly patients (age > 65 years) with HCCs ≤ 20 mm while surgical resection would be a better treatment for HCCs of 21-50 mm in elderly patients. A meta-analysis by *Hunget al*[38] found that

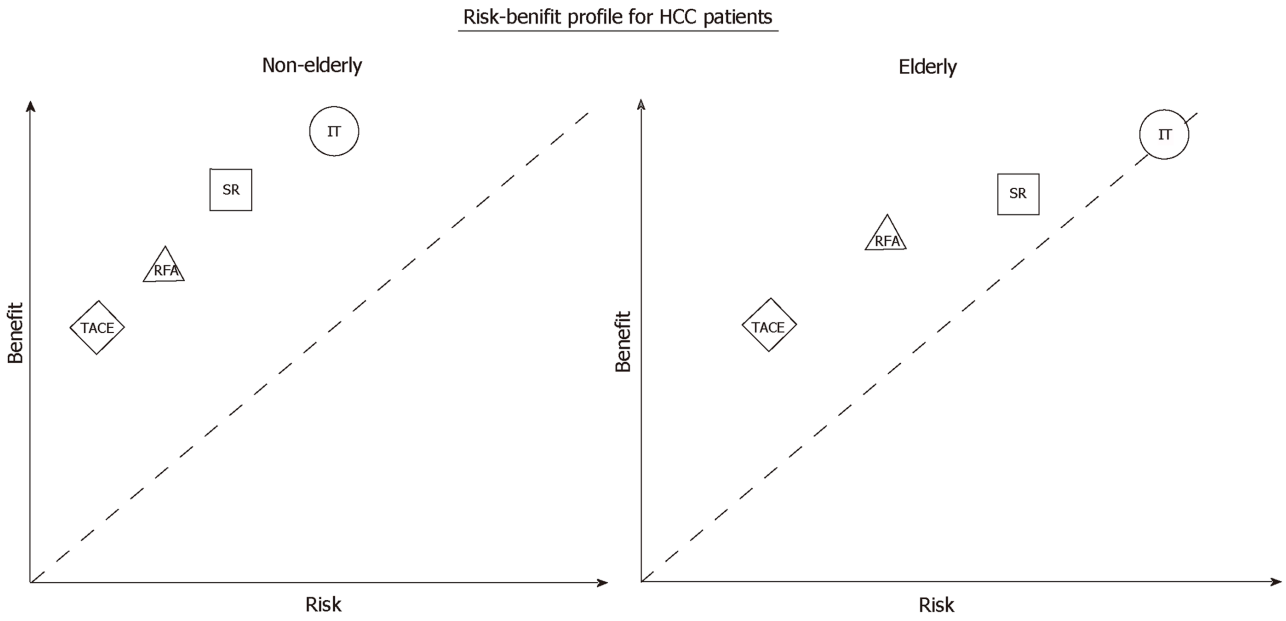

Figure 1 Schematic diagram–risk-benefit profile comparing aged patients with young patients in hepatocellular carcinoma treatments. TACE: Transarterial chemoembolization; RFA: Radiofrequency ablation; SR: Surgical resection; LT: Liver transplantation; HCC: Hepatocellular carcinoma.

elderly and young patients shared similar survival outcomes at one year and three years after RFA. However, the elderly group had a significantly worse 5-year survival rate (odds ratio = 1.379, P = 0.01). Unlike elderly patients having surgical resection, elderly patients having RFA had poorer survival when compared with young patients. This may be due to selection bias – elderly patients with a poorer general health profile were included into the RFA arm.

TRANSARTERIAL CHEMOEMBOLIZATION

TACE is a widely used nonsurgical treatment that is considered to be effective in prolonging HCC patients' survival[33,77-79]. It has been reported that development of peptic ulcer disease occurred in 2.5% of patients and development of liver failure occurred in 11% of patients[16]. The meta-analysis by Hung *et al*[38] found that elderly patients benefited more from TACE than younger patients did. Significantly better 1- and 3-year survival rates were seen in the elderly group (odds ratio = 0.664, P < 0.01 and odds ratio = 0.795, P = 0.013). Nonetheless, no significant difference in 5-year survival between the two groups of patients was observed. In general, elderly patients with HCC at an earlier stage or with a higher surgical risk are more likely to be selected to receive TACE. This may be a major reason to explain why some studies reported a better survival outcome in elderly patients after TACE.

TARGET THERAPY AND IMMUNOTHERAPY

Sorafenib represents the standard of care in the management of advanced HCC. It has been reported that sorafenib achieved similar progression-free survival and overall survival in elderly and young patients with advanced HCC[80]. However, morbidities including neutropenia, malaise and mucositis occurred more frequently in elderly patients. Dose reduction is a way to increase its tolerability[81,82]. Other common adverse effects (*e.g.*, hand and foot syndrome and diarrhea) were reported to be similar in both populations[80]. Certain novel immunotherapy agents have been approved and introduced into clinical practice to treat HCC, *e.g.*, anticytotoxic T lymphocyte-associated protein 4 antibody, anti-programmed cell death 1 antibody, and anti-programmed death-ligand 1 antibody. Despite limited data, immune checkpoint inhibitors may represent a potential option of nonsurgical treatment for elderly HCC patients[83].

CONCLUSION

Overall, elderly patients and younger patients benefited similarly from HCC treatments. Advanced age and comorbidity are intrinsic factors in elderly HCC patients but should not preclude them from receiving treatments. Patients should be evaluated individually and treatment options should be personalized. All treatment options available to the young should be made available to the elderly. Careful assessment of clinical status, cancer stage and comorbidity is needed to ensure good treatment outcomes. More clinical data are needed for the determination of selecting criteria on elderly HCC patients to maximize their chance of getting the most appropriate and effective treatments. As such, further studies evaluating the outcomes of different HCC treatment modalities in elderly patients are warranted.

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ORIGINAL ARTICLE

Basic Study

Mucosal healing progression after acute colitis in mice

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Abstract

BACKGROUND

Mucosal healing has become a therapeutic goal to achieve stable remission in patients with inflammatory bowel diseases. To achieve this objective, overlapping actions of complex cellular processes, such as migration, proliferation, and differentiation, are required. These events are longitudinally and tightly controlled by numerous factors including a wide range of distinct regulatory proteins. However, the sequence of events associated with colon mucosal repair after colitis and the evolution of the luminal content characteristics during this process have been little studied.

AIM

To document the evolution of colon mucosal characteristics during mucosal healing using a mouse model with chemically-induced colitis.

METHODS

C57BL/6 male mice were given 3.5% dextran sodium sulfate (DSS) in drinking water for 5 d. They were euthanized 2 (day 7), 5 (day 10), 8 (day 13), and 23 (day 28) d after DSS removal. The colonic luminal environment and epithelial repair processes during the inflammatory flare and colitis resolution were analyzed with reference to a non-DSS treated control group, euthanized at day 0. Epithelial repair events were assessed histo-morphologically in combination with functional permeability tests, expression of key inflammatory and repairing factors, and evaluation of colon mucosa-adherent microbiota composition by 16S rRNA sequencing.

RESULTS

The maximal intensity of colitis was concomitant with maximal alterations of

INRA (Comethea) and received the approval of the ministerial committee for animal experimentation (registration number: APAFIS#3987-2016012214388658), according to the European directive for the use and care of laboratory animals (2010/63/UE).

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intestinal barrier function and histological damage associated with goblet cell depletion in colon mucosa. It was recorded 2 d after termination of the DSS-treatment, followed by a progressive return to values similar to those of control mice. Although signs of colitis were severe (inflammatory cell infiltrate, crypt disarray, increased permeability) and associated with colonic luminal alterations (hyperosmolarity, dysbiosis, decrease in short-chain fatty acid content), epithelial healing processes were launched early during the inflammatory flare with increased gene expression of certain key epithelial repair modulators, including transforming growth factor-β, interleukin (Il)-15, Il-22, Il-33, and serum amyloid A. Whereas signs of inflammation progressively diminished, luminal colonic environment alterations and microscopic abnormalities of colon mucosa persisted long after colitis induction.

CONCLUSION
This study shows that colon repair can be initiated in the context of inflamed mucosa associated with alterations of the luminal environment and highlights the longitudinal involvement of key modulators.

Key words: Colon luminal environment; Dextran sodium sulfate-induced colitis; Dysbiosis; Epithelial repair; Acute colitis

Core tip: When colitis was chemically induced with dextran sodium sulfate, 2 d after the end of the treatment, mice showed unequivocal sign of colitis, changes in the luminal environment of the large intestine, epithelial permeability loss, and dysbiosis. These inflammation-induced alterations progressively and partly resolved in the period of time following colitis induction. Early and long-term evaluation of the epithelial repairing process showed overlapping action of inflammatory and repairing markers, rather than successive actions.

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INTRODUCTION

Inflammatory bowel diseases (IBD) are chronic inflammatory disorders of the gastrointestinal tract with unclear etiology. These pathologies display relapsing-remitting courses characterized by periods of inflammation and remission over long periods of time[1]. Maintaining stable remission without clinical symptoms and decreasing the incidence and duration of relapses are ultimate goals of IBD treatment[2]. A key step to achieving these objectives is the healing of inflamed mucosa, also known as mucosal healing (MH), described as the complete absence of blood, friability, erosion, and ulcerative lesions in all segments of the gut[3]. Indeed, clinical studies have shown the importance of MH on IBD outcomes, since MH is associated with lasting clinical remission, a decrease in intestinal complication risk, and less recourse to hospitalization and surgery[4].

The healing sequence starts with epithelial restitution, where epithelial cells surrounding the wounded area migrate to reseal the denuded basal membrane[5,6]. Within hours or days after injury, epithelial restitution is followed by additional steps in wound healing. These include increased epithelial cell proliferation to replenish the decreased cell pool, and later, maturation and differentiation of undifferentiated epithelial cells to recover colon physiological functions such as barrier function and water absorption[5,7]. These processes require tight control mechanisms that rely on modulation of numerous factors. They include a wide range of structurally distinct regulatory proteins, such as growth factors, cytokines, other peptide molecules like extracellular matrix (ECM) factors, and non-peptide molecules, including microbiota-derived metabolites[8]. Although their relevance as signal-transducers and modulators

of cell function, migration, proliferation and differentiation has been well established individually, mostly by *in vitro* studies[8,9], the concomitant interaction of these factors with epithelial repair *in vivo* has been less ascertained. Furthermore, although alterations of microbiota composition during acute colitis have been repeatedly observed in IBD patients[10-12], the impact of epithelial changes during MH on mucosa-adherent microbiota has barely been evaluated. This is despite the observation that it may have a major impact on the colonic epithelium due to its proximity to epithelial cells. Lastly, changes in the bacterial metabolite composition in the luminal fluid facing the colonic and rectal epithelium are also likely to influence the course of inflammatory processes[13].

Given the clinical relevance of MH for IBD patients, it is important to understand the sequence of events and the modulators that intervene in the healing progression as well as how these modifications may influence mucosa-adherent microbiota and, ultimately, the MH outcome. Our aim was to further the current understanding of MH progression after an acute inflammatory episode. We used a kinetics approach with complementary methods to study events involved in epithelial repair and mucosa-adherent microbiota restitution that contribute to the restoration of colon barrier function after an acute inflammatory challenge, which was chemically induced by dextran sodium sulfate (DSS) in mice.

MATERIALS AND METHODS

Animals and diets

Male C57BL/6 OlaHsd mice [n = 64, 7-week-old, body weight (BW) mean: 22.4 ± 0.2 g, Envigo, France] were acclimated for 1 wk with free access to standard mouse chow and tap water under controlled temperature (23 °C), humidity (55% ± 10%), and light (12:12-h light-dark cycle) conditions. Each mouse was maintained in an individual cage with a grid and acclimated to the diet after 3 d of a standard mouse chow/fresh P14 diet (Table 1). The study was performed according to the European directive for the use and care of laboratory animals (2010/63/UE) and received the approval of the local animal ethics committee and the ministerial committee for animal experimentation (registration number: APAFIS#3987-2016012214388658). Animals were randomly distributed in each group.

Experimental design

A normoproteic diet (P14, 140 g/kg milk protein) was used throughout the study (Table 1). Healthy controls (untreated mice at day 0, n = 12) received fresh tap water. DSS-treated mice (n = 52) received 3.5% (w/v) DSS (36000-50000 MW, MP Biomedicals Illkirch-Graffenstaden, France) in drinking water for 5 d, from day 1 to day 5 (fresh DSS solution being prepared every 2 d), to induce an acute episode of colitis[14]. Drink and food were provided *ad libitum*. DSS, food consumption, and BW were measured daily. Mice were evaluated using an inflammatory score based on stool consistency and rectal bleeding. Each of these elements was rated on a 0-3 scale, 0 representing no disease symptoms and 3 representing severe disease symptoms. Presented data are the sum of both scores. Long-term assessment of mice in the day 28 group was performed by dual-energy x-ray absorptiometry to measure body fat and lean body mass every 9 d using a PIXImus imager (GE Lunar PIXImus, GE Healthcare, Waukesha, WI, United States). In parallel, an *in vivo* permeability assay was performed on the day 28 group by gavage of 4 kDa paracellular marker fluorescein isothiocyanate-labeled dextran (FD4, Sigma-Aldrich, St. Louis, MO, United States). Food was withdrawn for 4 hours, and mice were gavaged with permeability tracer (0.6 mg/100 g BW of FD4). Plasma was collected from the tail 4 hours later. Plasmatic FD4-concentrations were determined by fluorescence measurement from a standard curve using serial dilutions of FD4 gavage solution. Mice were euthanized at day 10 (n = 12), 13 (n = 12), and 28 (n = 16) to evaluate the impact of diet on epithelial repair kinetics or if they lost more than 20% BW, as per approved animal protocol guidelines, to meet the end point criteria.

Tissue collection

At euthanasia, animals were anesthetized by isoflurane inhalation and euthanized by intracardiac puncture. Blood was collected in EDTA-containing tubes and plasma was frozen and kept at -80 °C for measurement of cytokines. The entire colon was removed, measured, and weighed. The colon was divided into six segments. One segment of the proximal colon was mounted in an Ussing chamber. Proximal colon mucosa was scraped, frozen in liquid nitrogen, and stored at -80 °C for subsequent analysis of adherent microbiota. Whole luminal colonic content was then removed for

Table 1 Composition of the experimental diet

Ingredient, g/kg	P14
Acid casein (Armor Protéines®, ref. 139860)	112
Whey protein (Armor Protéines®, Protarmor 80, ref. 139805)	28
Corn starch	622.4
Sucrose	100.3
Cellulose	50
Soybean oil	40
Mineral mixture (AIN 93-M)	35
Vitamin mixture (AIN 93-V)	10
Choline	2.3
Metabolizable energy, kJ/g	14.5

osmolarity and water content measurements. Another segment was harvested for RNA analysis and immediately frozen in TRIzol® Reagent (ThermoFisher Scientific, Waltham, MA, United States) and stored at -80 °C until further analysis. Two other segments were immediately frozen in liquid nitrogen and stored at -80 °C for myeloperoxidase (MPO) and protein expression assays. Two 1 cm segments of the distal colon were fixed in 4% buffered formaldehyde for histological analysis (longitudinal and transversal sections). Osmolarity of colonic content was measured with a freezing point depression osmometer (Löser Type 15 Roebling Osmometer, ThermoFisher Scientific), and water content was calculated as previously described[14].

Quantification of gene expression by real-time polymerase chain reaction (qRT-PCR)

Colonic samples were homogenized in TRIzol® Reagent using an ultra-turrax and phase separated. RNA was purified using an RNeasy Mini kit (Qiagen SAS, Courtabœuf, France) and DNase I treatment. qRT-PCR was performed with Fast SYBR Green MasterMix (Applied Biosystems, Foster City, CA, United States), gene-specific primers (sequences available on demand), and the StepOne Real-Time PCR system (Applied Biosystems, Life Technologies). Gene expression was determined using the $2^{-\Delta\Delta CT}$ formula, where $\Delta\Delta CT$ = (CT target gene - CT reference gene) using *Hprt* as the house-keeping gene and normalized to the day 7 group.

Determination of local and systemic inflammatory markers

Intestinal tissue was assayed for MPO activity as a neutrophil infiltration marker. Activity analysis was performed using an O-dianisidine dihydrochloride assay as previously described[15]. Colonic cytokine concentrations were measured in total colon protein lysate by Luminex technology using Bio-Plex kits[14] (Bio-Rad, Marnes-La-Coquette, France). Results were expressed as nanograms per milligram of total protein. Plasmatic concentration of lipopolysaccharide-binding protein was determined with commercial solid-phase sandwich enzyme-linked immunosorbent assay from Tebu-Bio (LBP, Pikoline Elisa Kit Mouse, Set220EK1274; Boechout, Belgium).

Histological analysis

Colonic sections stained with hematoxylin-and-eosin were coded for blind microscopic assessment by an external histological platform (Histalim, Montpellier, France), and microscopic changes were qualitatively described and scored using a severity scale (0 to 3). The histological score was calculated as the sum of the score of four criteria: goblet cell depletion, ulceration and/or erosion, percentage of crypt damage, and edema. Epithelial repair score was calculated as the sum of the score of gland hyperplasia and presence of mitotic cells, re-epithelialization, and crypt repair. The inflammatory infiltrate (increase in mononuclear cells and/or neutrophils) was also scored depending on the location: 1- mucosa (lamina propria), 2- submucosa, 3- muscularis or serosa. Images were digitalized using a slide scanner (Lamina, Perkin Elmer, Waltham, MA, United States) and the CaseViewer software v. 2.2 (3DHISTECH, Budapest, Hungary). Colonic length of well-oriented epithelial crypt, mucosae, sub-mucosae, and muscularis was determined by image analysis using Pannoramic Viewer software v. 1.15.4 (3DHISTECH, Budapest, Hungary). Periodic Acid Schiff staining was used to visualize mucus-producing cells on 4-μm transversal colon sections counterstained with hematoxylin.

Ussing chamber studies

Proximal colon samples, opened along the mesenteric line, were mounted in EasyMount Ussing chambers within 15 minutes of dissection (Physiologic Instrument Inc, San Diego, CA, United States) with an exposed area of 0.1 cm^2. Electric measurements were performed as previously described[16]. At the end of each procedure, tissue viability was assessed by adding the cholinergic drug carbachol (10^{-4} M) on the serosal side.

Evaluation of adherent mucosal microbiota composition

Total DNA was extracted from 20 mg of scraped mucosa samples using the PowerFecal DNA Isolation kit (MoBio Laboratories, Carlsbad, CA, United States) according to the manufacturer's protocol. DNA extracts were used for qRT-PCR analysis of the 16S ribosomal genes. Total bacteria were quantified by real-time qPCR using specific primers (HAD-1: 5'- TGGCTCAGGACGAACGCTGGCGGC -3' and HAD-2: 5'- CCTACTGCTGCCTCCCGTAGGAGT-3', annealing at 59 °C), Fast SYBR Green MasterMix (Applied Biosystems) and the StepOne Real-Time PCR system (Applied Biosystems, Life Technologies). A standard curve was generated from serial dilutions of a known copy number of the target gene cloned into a plasmid vector as previously described[17]. DNA were subjected to PCR amplification of the V3-V4 region of the 16S rDNA gene, and sequencing was performed at the GenoToul INRA platform (Castanet-Tolosan, France) using Illumina technology with MiSeq kit V2 2 x 250 bp. A total of 747538 high-quality sequences were produced in this study, with an average of 8100 reads per sample. Data analysis was performed as previously described[18].

Analysis of short chain fatty acids

The Kristensen *et al*[19] method was used to assay short chain fatty acids (SCFA) in cecal content. After bacterial metabolite extraction in supernatant, SCFA were derivatized by esterification and analyzed with a gas chromatograph equipped with a capillary column (30 m, 0.32 mm ID, RestekRtx 502.2) and fitted with a flame ionization detector. SCFA concentrations were determined by external standards with reference to internal standards.

Statistical analysis

The animal number for each group (n = 12) was the minimal number necessary to obtain statistically exploitable results according to the variables studied and the variability of the model. Results are expressed as means ± standard error. The mean of differential values for each time period was assessed by analysis of variance with Bonferroni *post-hoc* test. For repeated measurements, time was added as a repeated factor. All analyses were performed with RStudio software version 1.0.143 with packages lme4, car, and lsmeans. For all statistical tests, the level of significance was set to $P < 0.05$. Principal component analysis was performed at phylum and family-level taxonomy to assess the evolution of adherent-microbiota composition at day 0, 7, 10, 13, and 28. The analysis of similarity, conducted with the ANOSIM test, was used to assess the correlation between ecological distance (based on family composition) and time groups. 16S rDNA analysis was performed using R software and in-house pipeline as previously published[18].

RESULTS

Comprehensive follow-up of DSS-treated mice shows that the inflammation peak occurred 2 d after DSS removal with a progressive return to basal values

Administration of 3.5% DSS for 5 d induced severe BW loss (Figure 1A) and sharp diminution of the percentage of lean and fat body mass (Figure 1B) at day 7. Mice exposed to DSS experienced an average of 14% weight loss during the first week of DSS administration compared to their initial BW. Food intake follow-up showed a substantial decrease in dietary energy ingestion both during DSS-treatment and until day 7 (58%, $P < 0.001$) but then progressively increased to reach the baseline amount at day 17 (Figure 1A). The inflammatory score peaked at day 7 and diminished progressively to remain stable from day 15 until the end of the study. A total of 11 mice died or were euthanized because they reached endpoint criteria between day 2 and day 15. This inflammatory state was associated with intestinal barrier leakage that reached its maximum at days 6 and 9 and returned to its initial value at day 12 (Figure 1D). Altogether, follow-up and intestinal permeability data indicate that the maximal intensity of colitis appeared around day 7 (*i.e.* 2 d after DSS removal) and that inflammation resolution/epithelial repair occurred during the week following

the inflammation peak. To understand the succession of events involved in colon healing, they will be described kinetically at critical stages: every 3 d from the inflammatory peak (day 7), therefore, at day 10 and day 13; and 21 d in (day 28) to assess evolution of the inflammatory process and restoration of the intestinal basal state.

At the maximal intensity of colitis, severe colon histo-morphological changes are associated with major luminal environment alterations (day 7)

DSS impacts colon crypt architecture and permeability: Intestinal barrier permeability increase coincided with major changes of the colon structure, as evidenced by colon morphometry and histological analysis (Tables 2 and 3, Figure 2). DSS-treated colons were significantly shortened and thickened, as shown by the increase in the weight/length ratio (Table 2). The transmural potential of the proximal colon, measured in an Ussing chamber, was altered (-1.4 ± 0.4 mV in DSS-treated mice *vs* -4.7 ± 0.5 mV at day 0, $P < 0.05$). DSS treatment induced severe histological damage, as most mice presented with mucosal multifocal ulceration and/or erosion (Figure 2A) but with different degrees of severity according to the animals examined (severe for 5/11, moderate for 2/11, and minimal for 4/11 mice). All DSS-treated mice were subject to crypt disappearance associated with distension of the remaining crypts and cyst formation (large cystic spaces lined by flattened epithelial cells and filled with pale basophilic mucin) (Figure 2A). Unsurprisingly, DSS treatment induced goblet cell depletion (day 0: 13.36 ± 1.98 *vs* day 7: 4.95 ± 1.24 Periodic-acid Schiff-positive cells/well-oriented crypt, $P < 0.05$) and provoked modifications to their morphology. Goblet cell size was larger than usual, with abundant mucin-rich cytoplasm, peripheral nuclei, and open pole (Figure 2B). Accordingly, goblet cell markers were severely impacted by DSS (Table 4), such as the major intestinal secreted mucin *Muc2* and the related transcription factor *Klf4*. These histo-morphological changes were associated with an increased colonic permeability, as evidenced by plasmatic FD4 concentrations (Figure 1D) and a decreased expression of several tight-junction proteins. Gene expression of *Zonula Occludens 1* (*Tjp1*), Occludin (*Ocln*), and the permissive Claudin 2 (*Cldn2*) were indeed downregulated at day 7 compared to day 0 (Table 4).

DSS causes inflammatory cell recruitment: DSS-treated animals suffered from subacute to chronic and segmental or diffuse lesions accompanied by a moderate to severe increase in edema (clear spaces). These lesions affected the entire intestinal wall, with particular effects on mucosa and submucosa at day 7 compared to untreated mice at day 0 (Figure 2A and Table 3). DSS-treatment provoked mucosal and submucosal infiltration of mononuclear cells (Figure 2A), mostly diffusely distributed and multifocally extended to the serosa, particularly around the blood vessels. Simultaneously, there were neutrophil clusters within lamina propria and submucosa. Colon MPO activity was indeed markedly increased by a 5-fold factor compared to untreated mice (Table 3), indicating a massive neutrophil infiltration. Analysis of relative mRNA levels showed that pro-inflammatory cell recruitment was associated with a marked upregulation of inflammatory genes such as *Il-1β*, *Il-6*, *Tnf-a*, and cyclooxygenase-2 (*Ptgs* gene) compared to untreated mice (Table 4). Colon pro-inflammatory cytokine (IL-1β and IL-6) concentrations were accordingly higher than in untreated animals (Table 2). Moreover, this coincided with a tremendous, increased expression of genes that encode key enzymes and structural constituents involved in the ECM remodeling process at day 7 (Table 4). There was also an increased expression of cytokines involved in epithelial repair. Gene expression of *Il-22*, *Il-33*, and *Tgfβ-1* and *-3* were correspondingly upregulated compared to untreated animals, whereas *Il-13* and *Il-15* gene expressions were downregulated at day 7 after DSS-treatment (Table 4).

DSS-treatment alters luminal environment and induces microbiota dysbiosis: DSS intake induced luminal content hyperosmolarity and increased the percentage of water in colonic content (Table 2), consistent with the inflammatory score results (Figure 1). The cecal quantities of acetate, propionate, and butyrate (among the most important end products of bacterial fermentation) were significantly reduced at day 7 compared to day 0 (Table 5), while proportions between the three SCFA remained stable. These luminal environment modifications were associated with major changes in mucosa-adherent microbiota composition. While microbiota quantification showed an increase in total bacterial load (Table 5) after DSS-treatment, estimators of community richness (Chao) and diversity (Shannon) were markedly lower than those at day 0 (Table 5). In addition, the number of exclusive and shared species-level phylotypes were significantly reduced after inflammation (463.5 ± 31.4 at day 0 *vs* 318.1 ± 30.6 at day 7, $P = 0.002$). Furthermore, DSS-treatment heavily impacted the

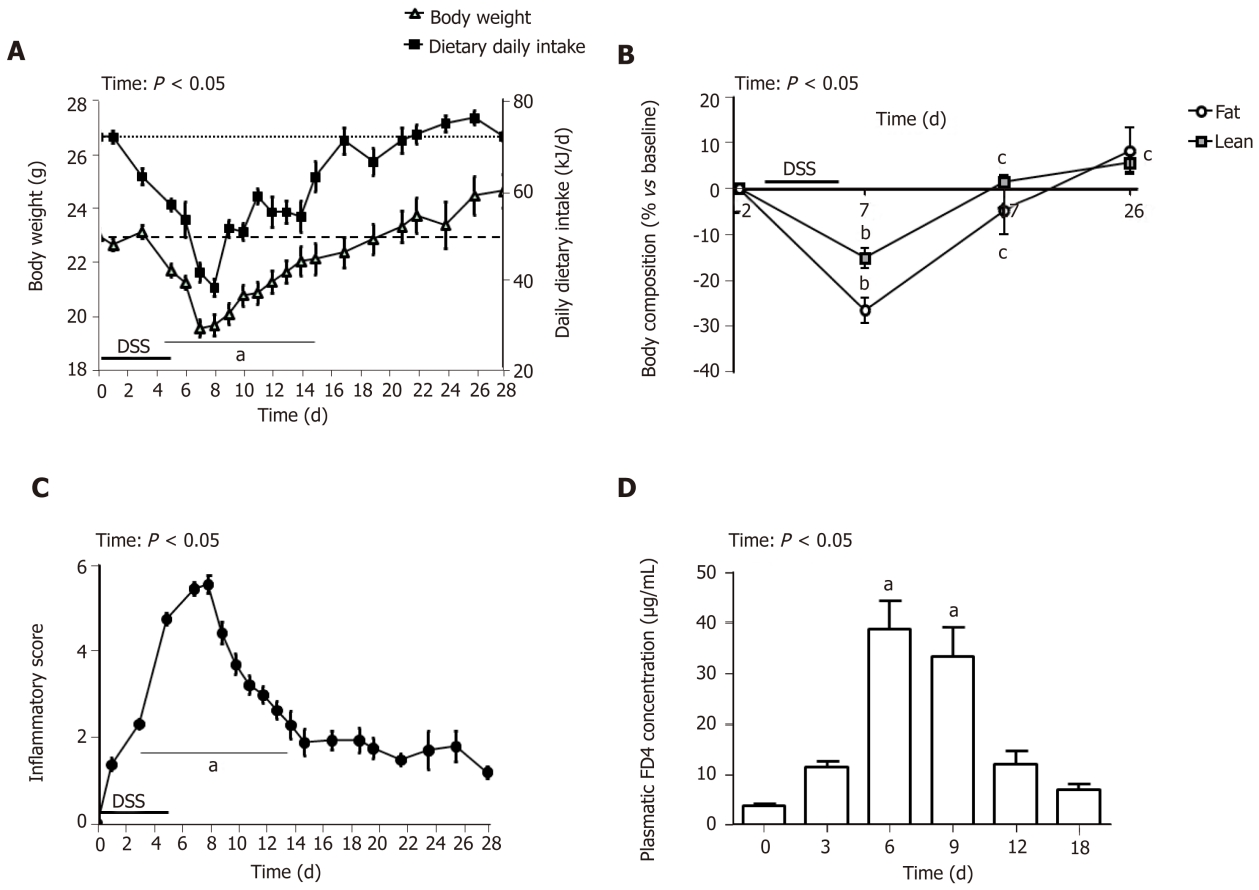

Figure 1 Follow-up parameters of dextran sodium sulfate-treated mice. A: Evolution of daily dietary intake and body weight; B: Evolution of lean and fat mass *versus* baseline; C: Evolution of the macroscopic inflammatory score; D: *In vivo* fluorescein isothiocyanate-dextran permeability measurement. Values are means ± SE (n = 8-12). [a]P < 0.05 *vs* day 0; [b]P < 0.05 *vs* day -2; [c]P < 0.05 *vs* day 7. FD4: Fluorescein Isothiocyanate-dextran; DSS: Dextran sodium sulfate.

mucosa-adherent microbiota at a higher taxonomic level, as shown by a decreased relative abundance of over 30 genera, the most relevant presented in Table 5. Colitis also impacted the phyla proportions: Bacteroidetes and Tenericutes decreased by 2 and 20-fold, respectively (P < 0.05); while Proteobacteria and Deferribacteres increased by 8 and 3-fold, respectively (P < 0.05) (Figure 3). Within the Firmicutes phylum, the *Eubacteriaceae*, *Lachnospiraceae*, and *Clostridiaceae* families (the latter including *Roseburia*, *Clostridium XIVa*, and *Butyrivibrio)* were significantly decreased following DSS treatment (Table 5). Conversely, within the Proteobacteria, DSS treatment led to a massive increase in the *Escherichia Shigella* proportion, accounting for increased representation of the *Enterobacteriaceae* family (Table 5).

Three days after the maximal intensity of colitis, epithelial repair occurs while signs of inflammation remain present (day 10)

Colon inflammation was still evident 5 d after DSS consumption arrest. At day 10, mice still suffered from severe ulceration and goblet cell depletion (Table 3). In one third of the animals, severe transmural inflammation continued to be observed with an increased gap between crypt bases and muscularis mucosae (Figure 2A), with a greater proportion of mononuclear cells. The neutrophils were mostly located at the edges of ulcerated areas showing epithelial exocytosis within the mucosa and/or crypt epithelium. In other animals, light to moderate inflammation was generally located within mucosa and mucosa/submucosa with an equilibrated proportion of mononuclear cells and neutrophils. Colonic MPO activity did indeed remain 4-fold greater than that in the untreated group, and IL-1β and IL-6 colonic concentrations were not different from day 7 (Table 2). However, gene expression of some pro-inflammatory markers (*Il-1β*, *Il-6*, *Ptgs*, *Tnf-a*) was decreased without reaching baseline values (Table 4). *In vivo* permeability increase continued to be observed at day 9 (Figure 1D) but was associated with a lower plasmatic concentration of the endotoxemic marker LBP when compared to day 7 (Table 2). This was concomitant and likely explained by increased gene expression of tight junction proteins that started to regain basal levels at day 10 (Table 4).

Table 2 Colon morphometric and inflammatory markers

Parameter	d0	d7	d7 *vs* d0	d10	d10 *vs* d7	d13	d13 *vs* d10	d28	d28 *vs* d0	Statistical effect (time)
Colon length (cm)	6.5 ± 0.2	4.9 ± 0.3[a]	↓	5.4 ± 0.2[a]	-	5.5 ± 0.2[a]	-	5.7 ± 0.2[a]	↓	< 0.001
Colon weight/length (mg/cm)	16 ± 0.3	24 ± 0.4[a]	↑	31 ± 3.0[ab]	↑	34 ± 3.0[ab]	-	38 ± 8.0[ab]	↑	< 0.05
Osmolarity of colonic content	180 ± 8	311 ± 15[a]	↑	278 ± 8.0[a]	-	288 ± 13[a]	-	281 ± 15[a]	↑	< 0.01
Colonic water content (%)	64.6 ± 2.5	87.5 ± 2.0[a]	↑	75.5 ± 3.0[ab]	↓	76.3 ± 1.5[ab]	-	74.0 ± 1.9[ab]	↑	< 0.001
IL-1β (ng/mg of total colon protein)	9.4 ± 1.8	14.3 ± 2.4[a]	↑	13.7 ± 1.8[a]	-	16.2 ± 2.5[a]	-	15.4 ± 2.2[a]	↑	< 0.01
IL-6 (ng/mg of total colon protein)	1.5 ± 0.5	3.6 ± 0.6[a]	↑	4.2 ± 0.8[a]	-	4.9 ± 1.2[a]	-	2.3 ± 0.3[bcd]	-	< 0.001
MPO activity (UA/mg total colon protein)	1.2 ± 0.2	5.8 ± 1.2[a]	↑	5.3 ± 0.8[a]	-	3.1 ± 0.8[abc]	↓	1.9 ± 0.6[bcd]	-	< 0.001
Plasmatic LBP (μg/mL)	2.0 ± 0.2	5.8 ± 0.4[a]	↑	4.5 ± 0.5[ab]	↓	4.9 ± 0.6[ab]	-	4.6 ± 0.3[c]	↑	< 0.001

C57BL/6 male mice were given 3.5% dextran sodium sulfate (DSS) for 5 d in their drinking water in order to induce an acute inflammatory episode. Colon morphometric and inflammatory markers were analyzed at peak colitis (day 7) and during colitis resolution (day 10, day 13 and day 28). Day 0 values correspond to non-DSS treated mice. Values are means ± SE (n = 8-12).

[a]$P < 0.05$ *vs* day 0;
[b]$P < 0.05$ *vs* day 7;
[c]$P < 0.05$ *vs* day 10;
[d]$P < 0.05$ *vs* day 13.

DSS: Dextran sodium sulfate; LBP: Lipopolysaccharide-binding protein; MPO: Myeloperoxidase activity.

First signs of epithelial repair are measured in a context of active inflammation: Despite a high inflammatory score, the healing process became visible while the thickening of the colon wall increased (Table 2). Sharp crypt hypertrophy accompanied by mild hyperplasia with increased mitotic figures was indeed observed at day 10 compared to day 7 (Figure 2A and Table 3). The edges of ulcerated areas showed minimal healing by re-epithelization, and edema was graded from moderated to minimal (Table 3). However, qRT-PCR data indicated that most factors that contribute to colon remodeling and epithelial repair had already been induced at day 7, since the expression of corresponding genes was maintained or reduced at day 10. The *Mmp7* gene expression was the notable exception with an almost 4–fold increase when compared to day 7 (Table 4).

First healing sequences are associated with major changes in microbiota-adherent mucosa composition: The osmolarity of luminal content remained high, while colon content was less watery at day 10 (Table 2). At that point, the three main SCFA concentrations were still severely diminished, notably the acetate concentration (Table 5). In addition, the relative abundance of the butyrate-producing *Clostridium cluster XIVa* species was significantly increased compared to day 7, reaching its initial proportion. Nonetheless, microbial dysbiosis remained present compared to day 7, as evidenced by similar proportions of Bacteroidetes, Deferribacteres, Tenericutes, and Proteobacteria phyla (Figures 3 and 4).

Six days after maximal intensity of colitis, epithelial repair is actively engaged even though inflammation is still active (day 13)

Colon DSS-induced inflammation diminishes in intensity: DSS-treated mice still

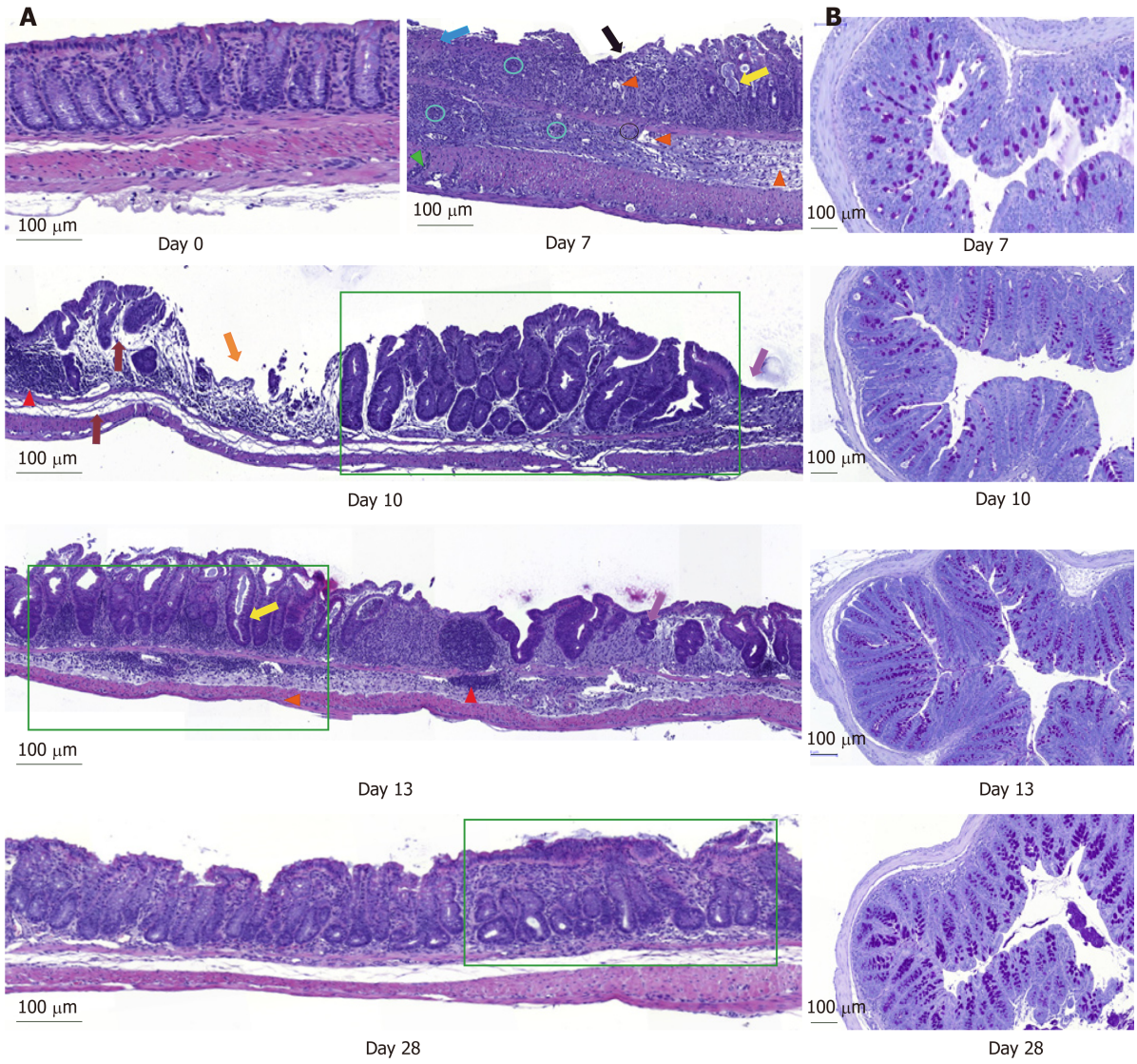

Figure 2 Histological examination of longitudinal and transversal colonic sections stained either with hematoxylin-and-eosin or with Periodic-acid Schiff. Magnification: 10 ×. A: Colon sections of the control mice at day 0 and dextran sodium sulfate-treated mice at days 7, 10, 13 and 28 (Blue arrow: Focal ulceration accompanied by fibrin; Circle: Clusters of neutrophils within mucosa and submucosa; Green arrow: Transmural increase of mononuclear cells; Pink arrow: Clear space due to of edema; Yellow arrow: Cyst formation; Red arrow: Mucosal lymphocytosis; Green rectangle: Crypt architectural disarray and crypt abscess; Grey arrow: Edema; Orange arrow: Multifocal ulceration; Violet arrow: Focal re-epithelization); B: Histological illustration of Periodic-acid Schiff staining during time. Scale bar: 100 μm.

presented with some crypt abscesses, but lesions were generally less severe than observed in the preceding days (although two animals still presented with a severe transmural leukocytic infiltrate) (Figure 2A). MPO activity was indeed significantly lower than at day 10, but remained higher than at day 0 (Table 2). Half of the mice showed healing by re-epithelization at the edges of ulcerated areas, and most animals showed marked architectural disarray and hyperplasia indicative of crypt regeneration (Figure 2A). These observations were confirmed by the epithelial repair score, which remained elevated at day 13 (Table 3). This was accompanied by improvement of both mechanical and chemical barrier functions, indicated by the increased mRNA expression of *Ocln* (Table 4) and goblet cell restoration (Figure 2B). Indeed, goblet cell depletion severity score was decreased (Table 3) in parallel with an increased expression of the goblet cell markers *Tff3* and *Muc2* (Table 4). The correlation analysis performed at day 13 also showed that *Tgf-β3*, *Il-15*, *Il-22*, and *Il-33* gene expressions were statistically correlated with the re-epithelization score (Figure 5). The initial *Il-15* colonic mRNA level was actually restored, and gene expression of colon *Saa* (serum amyloid A) was notably increased (Table 4). At day 12, the observed improvement of the intestinal barrier function (Figure 1D) may have been a consequence of these ongoing epithelial repair events.

Mucosa-adherent microbiota remains altered: The total biomass of mucosa-adherent

Table 3 Histological analysis scoring of inflammatory and healing parameters

Parameter	d7	d10	d10 *vs* d7	d13	d13 *vs* d10	d28	d28 *vs* d7	Statistical effect (time)
Inflammatory score	12.8 ± 0.8	9.3 ± 1.4[b]	↓	8.4 ± 1.3[b]	-	4.4 ± 1.7[bcd]	↓	< 0.001
Goblet cell depletion	2.27 ± 0.24	1.83 ± 0.21	-	1.08 ± 0.26[bc]	↓	0.88 ± 0.30[bc]	↓	< 0.001
Ulceration and erosion	2.09 ± 0.29	1.34 ± 0.31	-	1.17 ± 0.27	-	0.63 ± 0.32[bcd]	↓	< 0.001
Crypt damage	2.72 ± 0.19	1.58 ± 0.34[b]	↓	1.25 ± 0.30[b]	-	1.25 ± 0.45[b]	↓	< 0.001
Edema	2.09 ± 0.09	1.25 ± 0.22[b]	↓	1.42 ± 0.23[b]	-	0.0 ± 0.0[bcd]	↓	< 0.001
Inflammatory infiltrate (mononuclear cells)	1.91 ± 0.27	1.83 ± 0.30	-	1.75 ± 0.18	-	1.40 ± 0.33[bcd]	-	< 0.001
Inflammatory infiltrate (neutrophils)	1.73 ± 0.13	1.50 ± 0.29	-	1.75 ± 0.19	-	0.63 ± 0.32[bcd]	↓	< 0.001
Epithelial repair score	0.82 ± 0.22	2.83 ± 0.42[b]	↑	3.25 ± 0.43[b]	-	3.25 ± 0.25[b]	↑	< 0.001
Gland hyperplasia and presence of mitotic cells	0.0 ± 0.0	0.83 ± 0.24[b]	↑	0.92 ± 0.26[b]	-	0.88 ± 0.35[b]	↑	< 0.001
Re-epithelialization	0.54 ± 0.16	0.58 ± 0.15	-	0.58 ± 0.15	-	0.63 ± 0.33	-	NS
Crypt repair	0.27 ± 0.19	1.42 ± 0.34[b]	↑	1.75 ± 0.30[b]	-	1.75 ± 0.45[b]	↑	< 0.001

Hematoxylin-and-eosin stained colonic sections were coded for blind microscopic assessment by an external histological platform (Histalim, Montpellier, France), and microscopic changes were qualitatively described and scored using a severity scale (0 to 3). Measurements were performed at peak colitis (day 7) and during colitis resolution (day 10, day 13 and day 28). Values are means ± SE (n = 8-11).
[b]$P < 0.05$ *vs* day 7;
[c]$P < 0.05$ *vs* day 10;
[d]$P < 0.05$ *vs* day 13.
NS: Non-significant difference.

microbiota at day 13 was higher than at day 10. Proteobacteria and Deferribacteres relative percentages remained high at day 13, while Bacteroidetes relative abundance was reduced 3-fold (Figure 3), with a clear reduction in the *Bacteroidaceae* family (4-fold less, $P < 0.001$, Table 5). In contrast, the *Clostridium cluster XIVa* proportion had increased compared to day 10. However, cecal SCFA concentrations remained very low at that time (Table 5).

Three weeks after the maximal colitis intensity, epithelial repair is only partly achieved

DSS-treatment exerts long-term effects on colon crypt architecture and permeability: While numerous parameters associated with colon inflammation did reach baseline values (Tables 2-4), several DSS-induced abnormalities such as colon environmental changes (hyperosmolarity and watery content) persisted. Colon histomorphology also remained affected, as indicated by a high colon weight/length ratio (Table 2), crypt disarray (Figure 2A), and altered epithelium electrical parameters (transmural potential Vt: -1.2 ± 0.1 mV at day 28 *vs* -4.7 ± 0.5 mV at day 0, $P < 0.05$). Mice also still displayed inflammation traits, such as mild ulceration, mucosal erosion (Table 3), and increased gene expression of *Tnf-a*. Furthermore, higher concentrations of IL-1β in the colon, and of the endotoxemic marker in plasma, were recorded in DSS-treated mice when compared to untreated mice (Table 2). It is noteworthy that colon *Il-33* and *Saa* gene expression remained elevated in DSS-treated mice (Table 4).

Inflammation alters mucosa-adherent microbiota in the long term: At day 28, dysbiosis was still noticeable, as evidenced by principal component analysis at both phylum and family levels. The different sample clustering between day 0 and day 28 highlighted a distinct microbial structure impairment (Figure 4). Indeed, the total bacterial load associated with the mucosa was slightly but significantly higher than at day 0, while both diversity and community richness indexes remained reduced (Table 5). In addition, while the cecal SCFA had returned to baseline proportions at day 28, the absolute concentrations of these metabolites were 3-fold lower than at day 0 (Table 5). Tenericutes and Actinobacteria were the only two phyla for which a significant difference was still observed at day 28. Proportions of *Prevotellaceae* and

Table 4 Kinetics of colonic mRNA expression level normalized to day 7 group (2-ΔΔCt)

	d0	d7	d7 *vs* d0	d10	d10 *vs* d7	d13	d13 *vs*d10	d28	d28 *vs*d0	Statistical effect (time)
Tight-junction protein										
Cldn2	2.41 ± 0.29	1.24 ± 0.21[a]	↓	2.90 ± 0.15[b]	↑	1.94 ± 0.17[b]	-	2.26 ± 0.31[b]	-	< 0.001
Tjp1	1.45 ± 0.09	1.03 ± 0.08[a]	↓	1.17 ± 0.10[b]	↑	1.30 ± 0.06[b]	-	1.29 ± 0.06[b]	-	< 0.01
Ocln	2.92 ± 0.37	1.18 ± 0.24[a]	↓	1.36 ± 0.11[a]	-	1.96 ± 0.18[abc]	↑	2.21 ± 0.30[bc]	-	< 0.001
ECM remodeling										
Mmp7	0.58 ± 0.16	1.46 ± 0.40[a]	↑	5.31 ± 1.37[ab]	↑	5.21 ± 1.22[ab]	-	3.69 ± 0.85[ab]	-	< 0.001
Mmp9	0.13 ± 0.01	1.35 ± 0.24[a]	↑	1.17 ± 0.27[a]	-	0.89 ± 0.15[a]	-	0.55 ± 0.20[bcd]	-	< 0.001
Timp1	0.05 ± 0.00	1.75 ± 0.49[a]	↑	0.84 ± 0.25[ab]	↓	0.41 ± 0.05[b]	-	0.23 ± 0.06[bc]	-	< 0.001
Col3a 1	0.77 ± 0.07	1.60 ± 0.33[a]	↑	1.15 ± 0.15[a]	-	1.20 ± 0.12[a]	-	0.83 ± 0.16[bcd]	-	< 0.01
Wisp	0.31 ± 0.02	1.40 ± 0.35[a]	↑	0.78 ± 0.17[ab]	↓	0.52 ± 0.04[b]	-	0.43 ± 0.06[b]	-	< 0.001
Acta2	1.33 ± 0.17	1.25 ± 0.22	-	0.95 ± 0.16	-	0.79 ± 0.05[a]	-	0.85 ± 0.11	-	< 0.01
Goblet cell markers and mucins										
Tff3	1.80 ± 0.24	1.29 ± 0.32	-	1.05 ± 0.12[ad]	-	1.62 ± 0.19[c]	↑	1.87 ± 0.13[c]	-	< 0.05
Muc2	3.77 ± 0.64	1.10 ± 0.16[a]	↓	1.72 ± 0.22[a]	-	3.03 ± 0.18[bc]	↑	3.38 ± 0.21[bc]	-	< 0.001
Klf4	2.31 ± 0.13	1.12 ± 0.22[a]	↓	1.52 ± 0.15[a]	-	1.73 ± 0.10[a]	-	2.56 ± 0.24[bcd]	-	< 0.001
Epithelial repair modulating factors										
Igf-1	0.27 ± 0.02	1.23 ± 0.19[a]	↑	1.14 ± 0.22[ad]	-	0.77 ± 0.06[ac]	-	0.55 ± 0.09[abcd]	-	< 0.001
Il-15	4.59 ± 0.89	1.32 ± 0.51[a]	↓	1.67 ± 0.18[a]	-	3.61 ± 0.91[bc]	↑	4.73 ± 0.86[bc]	-	< 0.01
Il-22	0.33 ± 0.20	1.59 ± 0.44[a]	↑	0.36 ± 0.08[b]	↓	0.47 ± 0.14[b]	-	0.18 ± 0.04[b]	-	< 0.001
Il-33	0.08 ± 0.02	1.69 ± 0.43[a]	↑	0.60 ± 0.11[ab]	↓	0.43 ± 0.05[ab]	-	0.26 ± 0.06[ab]	↑	< 0.001
Saa	1.51 ± 0.27	1.21 ± 0.23	-	1.05 ± 0.16	-	3.40 ± 0.40[abc]	↑	3.18 ± 0.31[abc]	↑	< 0.001
Tgf-ß1	0.50 ± 0.03	1.29 ± 0.28[a]	↑	0.72 ± 0.07[b]	↓	0.51 ± 0.07[b]	-	0.43 ± 0.04[b]	-	< 0.001
Tgf-ß3	1.08 ± 0.11	1.47 ± 0.37	-	0.72 ± 0.10[b]	↓	0.93 ± 0.12	-	1.16 ± 0.16	-	< 0.01
Inflammatory markers										
Il-1ß	0.04 ± 0.01	1.71 ± 0.50[a]	↑	0.39 ± 0.06[ab]	↓	0.45 ± 0.07[ab]	-	0.39 ± 0.09[ab]	↑	< 0.001
Il-6	0.01 ± 0.00	1.73 ± 0.51[a]	↑	0.84 ± 0.28[ab]	↓	0.41 ± 0.08[b]	-	0.15 ± 0.07[b]	-	< 0.001
Il-10	0.23 ± 0.03	1.37 ± 0.27[a]	↑	0.37 ± 0.04[b]	↓	0.31 ± 0.06[b]	-	0.25 ± 0.03[b]	-	< 0.001
Il-13	3.81 ± 0.75	1.26 ± 0.23[a]	↓	0.82 ± 0.08[a]	-	0.78 ± 0.10[a]	-	2.53 ± 0.39[bcd]	-	< 0.001
Tnf-a	0.13 ± 0.01	1.29 ± 0.22[a]	↑	0.46 ± 0.05[ab]	↓	0.50 ± 0.06[ab]	-	0.47 ± 0.07[ab]	↑	< 0.001
Ptgs	0.14 ± 0.01[a]	1.69 ± 0.39[b]	↑	0.84 ± 0.30[c]	↓	0.60 ± 0.10 c	-	0.29 ± 0.05[a]	-	< 0.001

Values are means ± SE (n = 8-12). Means with different superscripts within a row are significantly different.
[a]P < 0.05 *vs* day 0;
[b]P < 0.05 *vs* day 7;
[c]P < 0.05 *vs* day 10;
[d]P < 0.05 *vs* day 13. ECM: Extracellular matrix.

Rikenellaceae families (both from the Bacteroidetes phylum), *Clostridiaceae*, *Eubacteriaceae* and *Lachnospiraceae* (Firmicutes phylum), and *Anaeroplasmataceae* (Tenericutes phylum) remained reduced. Conversely, both Actinobacteria phylum (Figure 3) and *Bacteroidaceae* family proportions were strongly increased compared to day 0 (Table 5).

DISCUSSION

The present study examined the kinetics of molecular and cellular events in association with mucosa-adherent microbiota modifications involved in epithelial repair after acute colon inflammation induced by DSS administration. This study highlighted the heterogeneous responsiveness to DSS and evidenced long-term DSS-induced alterations on the colon luminal environment, notably on adherent microbiota composition, with associated changes in colon histo-morphology. This research also showed that epithelial healing processes were launched early during the inflammatory flare, supporting the concept of intricate involvement of certain key colon factors (Tgf-β, Il-15, Il-22, and Il-33) on epithelial repair modulation. Such

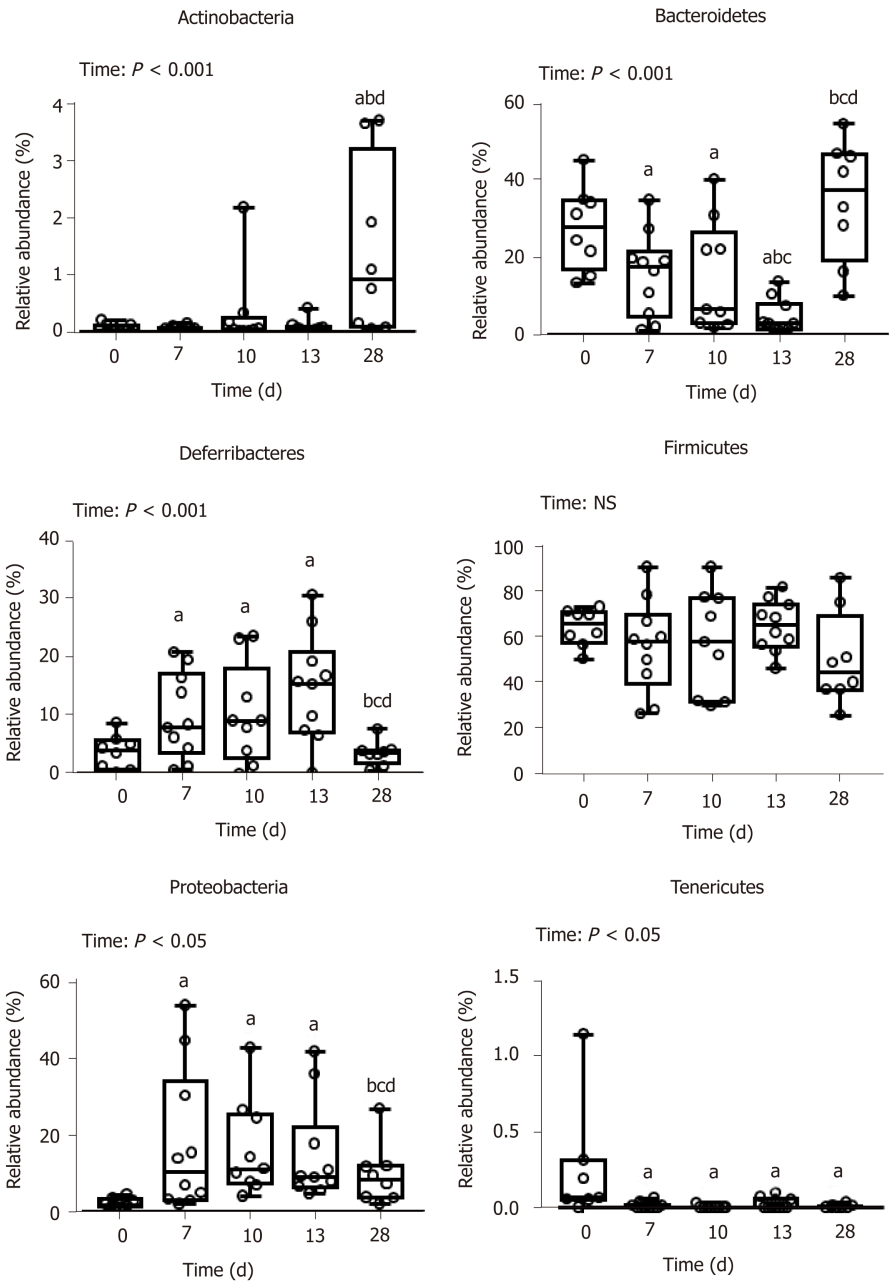

Figure 3 Phylum-relative abundance of mucosa-adherent microbiota. Values are means ± SE (n = 8-12). Means with different superscripts are significantly different. [a]P < 0.05 *vs* day 0; [b]P < 0.05 *vs* day 7, [c]P < 0.05 *vs* day 10; [d]P < 0.05 *vs* day 13. SE: Standard error; NS: Non-significant difference.

factors could be potential therapeutic targets for MH enhancement. Few studies have investigated the mucosa healing signature after an acute intestinal inflammatory flare. Previous studies have rather focused on the evolution of the inflammation markers[20] than on the repairing factors. In addition, most studies were carried out over a shorter period of time after colitis induction[21-23].

DSS-induced colitis is one of the most commonly used animal models of IBD, reflecting many clinical features of ulcerative colitis, such as infiltration of inflammatory cells, crypt loss and erosion, local production of MPO, and inflammatory cytokines in the distal colon[24-27]. This model is generally described as reproducible once the mouse strain and DSS dose/duration have been defined[27-29]. However, this qualitative histological analysis demonstrated relatively high variability in DSS responsiveness among animals, in contrast to other studies[21,23,30]. The reasons for such heterogeneity between animals are not known, but it is tempting to speculate that it is related to animals having been individually housed in this study (in order to accurately follow the inflammatory score and food consumption). Such conditions may amplify differences in the microbiota composition between animals[31]. With this in mind, this study clearly shows that DSS treatment induced common

Table 5 Mucosa-adherent microbiota composition and cecal bacterial metabolic activity

Parameter		d0	d7	d7 *vs* d0	d10	d10 *vs* d7	d13	d13 *vs* d10	d28	d28 *vs* d0	Statistical effect (time)
Total bacteria (log/g mucosal content)		9.36 ± 0.35	10.4 ± 0.14[a]	↑	9.92 ± 0.16[b]	↓	10.6 ± 0.22[ac]	↑	10.2 ± 0.24[ac]	↑	< 0.001
Shannon index		6.46 ± 0.07	4.75 ± 0.32[a]	↓	4.56 ± 0.28[a]	-	4.44 ± 0.26[a]	-	5.03 ± 0.29[a]	↓	< 0.001
Chao index		794 ± 55.2	633 ± 43.7[a]	↓	593 ± 31.1[a]	-	635 ± 34.4[a]	-	606 ± 51.6[a]	↓	< 0.01
Actinobacteria (%)											
Bifidobacteriaceae		0.02 ± 0.01	0.01 ± 0.00	-	0.01 ± 0.01	-	0.00 ± 0.00	-	0.60 ± 0.28[abcd]	↑	< 0.001
Coriobacteriaceae		0.01 ± 0.02	0.04 ± 0.02	-	0.07 ± 0.04	-	0.06 ± 0.04	-	0.83 ± 0.31[abcd]	↑	< 0.001
Bacteroidetes (%)											
Bacteroidaceae		4.35 ± 1.24	5.6 ± 1.34	-	11.55 ± 2.77[b]	↑	3.08 ± 1.08[c]	↓	20.1 ± 4.98[cd]	↑	< 0.05
Porphyromonadaceae		12.1 ± 2.55	7.40 ± 2.08	-	2.53 ± 1.29	-	1.03 ± 0.35[ab]	-	12.1 ± 5.18[d]	-	< 0.001
Prevotellaceae		1.06 ± 0.33	0.01 ± 0.00[a]	↓	0.01 ± 0.01[a]	-	0.02 ± 0.01[a]	-	0.01 ± 0.01[a]	↓	< 0.01
	Alloprevotella	1.04 ± 0.32	0.00 ± 0.00[a]	↓	0.01 ± 0.01[a]	-	0.01 ± 0.01[a]	-	0.00 ± 0.00[a]	↓	< 0.05
Rikenelleceae		9.64 ± 1.79	1.39 ± 0.61[a]	↓	0.33 ± 0.24[a]	-	0.31 ± 0.26[a]	-	2.04 ± 1.20[a]	↓	< 0.001
	Alistipes	9.48 ± 0.72	1.04 ± 0.51[a]	↓	0.24 ± 0.15[a]	-	0.13 ± 0.09[a]	-	1.98 ± 1.20[a]	↓	< 0.001
Deferribacterres (%)											
Deferribacteraceae	*Mucispirillum*	3.53 ± 1.86	9.98 ± 2.33[a]	↑	10.16 ± 3.04[a]	-	14.82 ± 2.80[a]	-	3.49 ± 0.76[bcd]	-	< 0.001
Firmicutes (%)											
Clostridiaceae	*Roseburia*	0.80 ± 0.17	0.01 ± 0.00[a]	↓	0.01 ± 0.01[a]	-	0.01 ± 0.01[a]	-	0.03 ± 0.01[a]	↓	< 0.001
	Clostridium XIVa	6.07 ± 0.84	1.09 ± 0.35[a]	↓	5.56 ± 2.73[b]	↑	8.54 ± 2.80[abc]	↑	4.01 ± 1.64[b]	-	< 0.05
	Butyrivibrio	0.28 ± 0.21	0.00 ± 0.00[a]	↓	0.00 ± 0.00[a]	-	0.00 ± 0.00[a]	-	0.00 ± 0.00[a]	↓	< 0.01
Eubacteriaceae		0.17 ± 0.05	0.04 ± 0.02[a]	↓	0.00 ± 0.00[a]	-	0.00 ± 0.00[a]	-	0.00 ± 0.00[a]	↓	< 0.001
Lachnospiraceae		35.0 ± 4.85	13.5 ± 2.91[a]	↓	21.7 ± 4.25[a]	-	23.2 ± 4.49[a]	-	23.6 ± 4.69	-	< 0.01
	Acetitafactor	5.46 ± 1.47	1.28 ± 0.55[a]	↓	0.30 ± 0.13[a]	-	1.11 ± 0.63[a]	-	1.00 ± 0.63[a]	↓	< 0.001
Lactobacillaceae		0.42 ± 0.19	0.24 ± 0.12	-	3.41 ± 2.82	-	0.63 ± 0.26	-	1.44 ± 0.88	-	NS
Ruminococcaceae		24.7 ± 4.5	30.4 ± 6.4	-	18.2 ± 3.6[b]	↓	26.7 ± 4.0	-	21.1 ± 4.0	-	< 0.05
Proteobacteria (%)											
Desulfovibrionaceae		1.94 ± 0.39	1.68 ± 0.32	-	3.06 ± 0.64	-	3.64 ± 0.95[ab]	-	1.30 ± 0.31[d]	-	< 0.01
Enterobacteriaceae		0.24 ± 0.04	14.38 ± 5.93[a]	↑	6.28 ± 2.59[a]	-	5.99 ± 3.80[a]	-	0.55 ± 0.16[bcd]	-	< 0.01
	Escherichia Shigella	0.05 ± 0.03	14.08 ± 5.83[a]	↑	6.02 ± 2.53[a]	-	5.47 ± 3.46[bc]	-	0.30 ± 0.11[bc]	-	< 0.01
Pseudomonadaceae		0.11 ± 0.04	0.06 ± 0.01	-	0.06 ± 0.02	-	0.18 ± 0.09	-	0.21 ± 0.12	-	NS
Sutterellaceae		0.05 ± 0.40	1.02 ± 0.42	-	2.87 ± 0.87	-	4.97 ± 1.93	-	7.39 ± 2.97	-	NS
Rhodospirillaceae		0.14 ± 0.05	0.51 ± 0.26[a]	↑	0.02 ± 0.02[b]	↓	0.00 ± 0.00[b]	-	0.04 ± 0.02[b]	-	< 0.001
Tenericutes (%)											
Anaeroplasmataceae		0.23 ± 0.13	0.00 ± 0.00[a]	↓	0.00 ± 0.00[a]	-	0.01 ± 0.01[a]	-	0.00 ± 0.00[a]	↓	< 0.01
Total SCFA (μmol/g)		12.4 ± 1.63	1.13 ± 0.78[a]	↓	0.06 ± 0.04[a]	-	0.14 ± 0.07[a]	-	4.88 ± 1.08[abcd]	↓	< 0.001
Acetate		8.95 ± 1.16	0.94 ± 0.67[a]	↓	0.04 ± 0.04[ab]	↓	0.14 ± 0.07[ab]	-	3.40 ± 0.71[abcd]	↓	< 0.001
Propionate		1.93 ± 0.29	0.04 ± 0.04[a]	↓	0.00 ± 0.00[a]	-	0.00 ± 0.00[a]	-	0.90 ± 0.26[abcd]	↓	< 0.001
Butyrate		1.51 ± 0.22	0.15 ± 0.11[a]	↓	0.01 ± 0.01[a]	-	0.00 ± 0.00[a]	-	0.59 ± 0.13[abcd]	↓	< 0.001

Quantification of total bacteria in the mucosa adherent microbiota was determined by real-time qPCR. Values are expressed as log of gene copy numbers of the different bacterial groups per gram of mucosal content. Evolution of the relative abundance of mucosa-adherent microbiota families was assessed by Illumina MiSeq sequencing. Cecal short chain fatty acids concentrations were obtained by gas chromatography after esterification. Values are means ± SE (n = 8-12).

[a]$P < 0.05$ *vs* day 0;

[b]$P < 0.05$ *vs* day 7;

[c]$P < 0.05$ *vs* day 10;

[d]$P < 0.05$ *vs* day 13.

NS: Non-significant difference; SCFA: Short chain fatty acids.

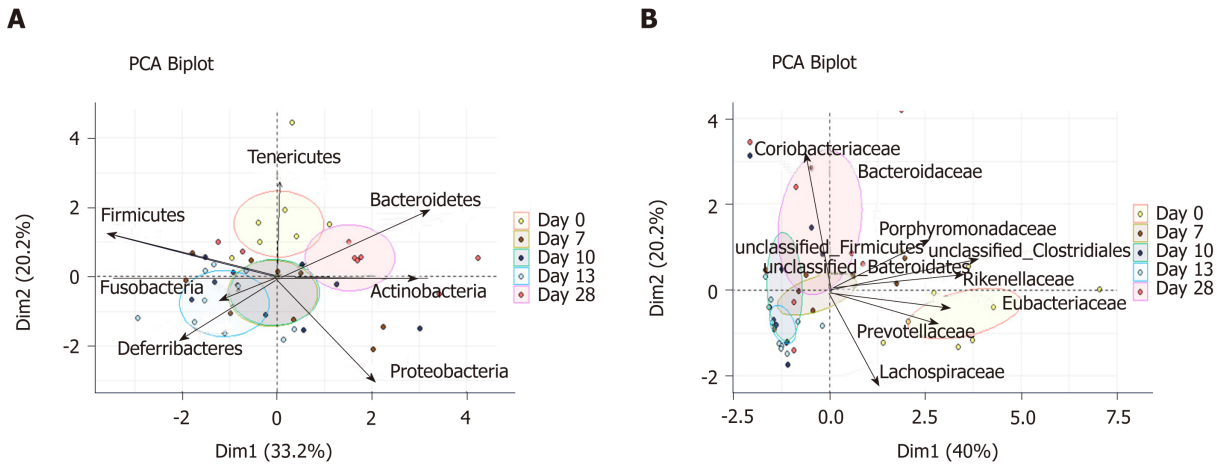

Figure 4 Principal component analysis of relative abundances of mucosa-adherent microbiota. A: Phylum level; B: Family level. Partial sample clustering by time was observed. PCA: Principal component analysis.

modifications of luminal content physicochemical parameters, such as osmolarity, water, and short-chain fatty acid luminal contents.

Major changes in mucosal-associated microbiota at the peak of colitis and during the days after may have resulted from a luminal increase in oxygen concentration caused by blood presence, in turn induced by inflammatory lesions. For instance, the increased bacterial biomass at mucosal sites at day 7 may be the consequence of an increase in redox potential that promoted development of facultative anaerobic bacteria such as Proteobacteria[32]. At day 10, relative abundance of *Lachnospiraceae* and *Ruminococcaceae* was reduced, similar to the microbial dysbiosis observed in IBD patients[33-35]. *Eubacteriaceae* and *Clostridiaceae* showed no improvement compared to proportions found in mice euthanized at day 7. This was particularly true for the *Roseburia* and *Butyrivibrio* genera, which are generally considered beneficial due to their role in butyrate production. Meanwhile, the proportion of *Escherichia Shigella* (Proteobacteria), strongly linked to intestinal inflammation[36], increased until day 13. These taxonomic shifts replicated what is generally observed in IBD patient microbiota[11,34,37,38], characterized by chronic dysbiosis even during remission[39]. These results did indeed show a severe colitis-associated dysbiosis of mucosa-associated microbiota, with an α-diversity loss that persisted throughout the resolution phase (from day 10 to day 28). As in ulcerative colitis, where longitudinal variations in mucosal bacterial populations are associated with disease severity[40], depletion of these bacterial families may be linked to observed functional disturbances during the resolution phase, such as altered epithelial barrier and inflammatory flare[41]. The drastic and persistent reduction of SCFA concentrations in the cecum was also likely due to disturbance of the cecal microbiota composition and/or metabolic activity, thus altering SCFA production. Acute lesions in the cecum induced by DSS have been described[42,43], though it is not one of the most common features[27].

This work indicates two important findings: Epithelial repair induction started rapidly even while inflammation was still severe; and although permeability was largely restored 6 d after the inflammatory peak, colon alterations persisted long after, likely because of consequential tissue remodeling. This is supported by observation of the over-expression of inflammatory markers and ECM remodeling factors as well as factors that influence epithelial repair, namely Tgfβ[44], Il-22[45], Il-33[46], and Igf-1[47,48]. This study showed that Tgf-β3, Il-22, Il-33, and Il-15, but not Igf-1, positively correlated with the re-epithelization score at day 13. The expression of Il-33 remained higher than in control animals throughout the experiment; it may, in particular, be a promising therapeutic target in the MH process. While conflicting results were obtained regarding its role in colitis, IL-33 has recently been shown to attenuate colitis and to favor colon repair in mice by promotion of M2 macrophage development and stimulation of goblet cell differentiation[49]. Moreover, the acute phase protein SAA, usually analyzed in plasma, displayed an increased gene expression in the colon 6 d after the maximal intensity of colitis. Importantly, SAA was recently identified as a protective factor against colon epithelium acute injury[50].

Several results obtained in this study regarding Il-15 expression deserve additional attention. This interleukin is considered a deleterious factor in cases of colitis[51]. IL-15 actually promotes intestinal dysbiosis associated with butyrate deficiency and increased susceptibility to colitis[52]. Although severely decreased in this study in the

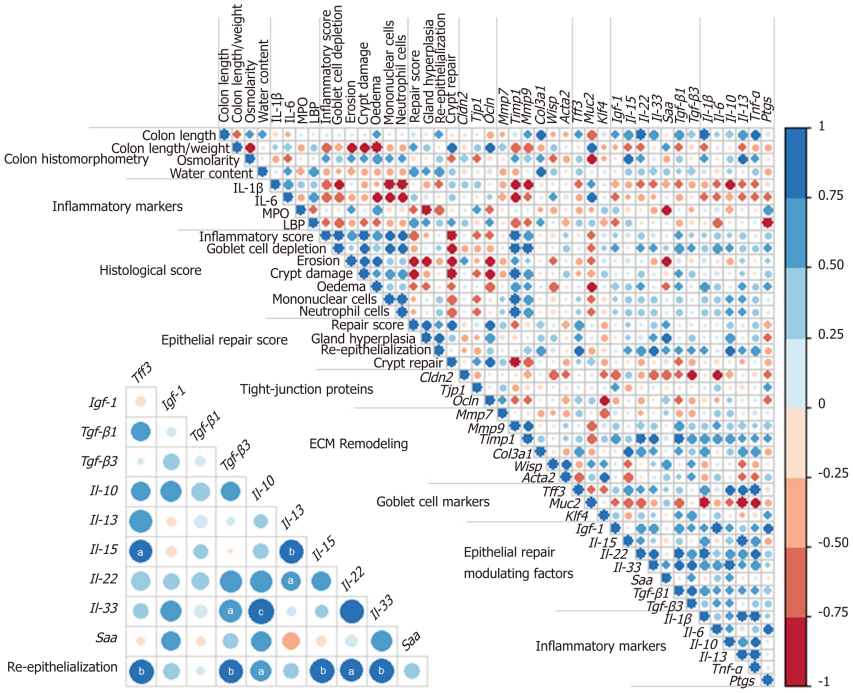

Figure 5 Correlation matrix of analytical parameters involved in inflammation and repair at day 13. Color intensity represents the degree of association between the parameters as measured by Spearman's correlations. Blue color indicates positive association and red color negative association. Lower triangular matrix corresponds to associations between histological re-epithelialization score and gene expression value of mucosal repairing factors. Statistically significant correlations are indicated with $^{a}P < 0.05$, $^{b}P < 0.01$ $^{c}P < 0.001$. (n = 8-12). ECM: Extracellular matrix; LBP: Lipopolysaccharide-binding protein; MPO: Myeloperoxidase activity.

course of colitis induction, IL-15 followed the same expression kinetics as those of epithelial repair, this process being characterized by the restoration of goblet cells and expression of genes coding for tight-junction proteins. Notably, in other pathological contexts, this cytokine has been shown to promote wound repair *via* TGFβ[53] and IGF-1[54] production, suggesting that IL-15 may be a therapeutic agent to manage wound healing. Additional, specific immunohistochemical assays, outside the scope of the present study, are necessary to identify which cells produce these modulating epithelial repair factors, since they may be synthesized by many cell types in the colon mucosa (colonocytes, innate cells, macrophages, myofibroblasts, or fibroblasts). Unsurprisingly, epithelial repair coincides with goblet cell restoration, as evidenced in this study by *Muc2* expression increase and a decreased goblet cell depletion score. This was associated with increased expression of gene coding for the major epithelial repair factor *Tff3* 6 d after the peak colitis. However, this also indicates that in this model, epithelial repair was triggered by pathways independent of Tff3 since histological improvements had already been observed 3 d before changes in *Tff3* expression.

Overall, this histo-morphological analysis, the measurement of several inflammatory markers, the luminal parameters related to the microbiota composition, and SCFA concentrations indicate effects of DSS on colon mucosa that lasted more than 3 wk after DSS removal from drinking water. Although MH is usually considered attained when lesions are no longer visible in the colon after an inflammatory flare, microscopic colon mucosa abnormalities were still present 3 wk after the colitis peak in this study. These alterations might be related to residual inflammatory infiltrate with high expression of pro-inflammatory cytokines (Il-1β and Tnf-α) and Mmp7, whereas other factors involved in ECM remodeling returned to their basal levels. Future studies using an Mmp-7 inhibitor would be useful to evaluate the involvement of this potentially therapeutic modulator in colitis progression[55]. Finally, the resulting mild colon mucosa disarray associated with microscopic inflammation raises questions about clinical consequences for patients without endoscopic lesions, specifically in terms of relapse risk and/or associated complications.

In conclusion, the present study showed the longitudinal evolution of several key parameters associated with inflammation and healing during and after inflammatory flare in a DSS model of colitis. As previously proposed[56], our study offers additional

information regarding the imbrication of mucosal inflammation and repairing processes. Such a concept is worth considering for further research aiming at improving intestinal MH by means of therapeutic and nutritional interventions.

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ARTICLE HIGHLIGHTS

Research background

Rapid mucosal healing after an intestinal inflammatory episode is considered beneficial to diminishing the relapse risk in inflammatory bowel disease patients. However, the event progression of colon mucosal repair after a colitic flare has barely been studied.

Research motivation

A better understanding of the events associated with inflammation resolution and mucosal healing are necessary to identify potential targets for colon mucosal healing enhancement.

Research objective

To document longitudinal modifications of the colon mucosa and luminal ecosystem following an episode of chemically-induced colitis.

Research methods

Evolution of colon mucosa inflammation and healing indicators, as well the changes in colonic luminal environment, were assessed in dextran sodium sulfate-treated mice during the 3 wk after the maximal intensity of colitis. Complementary approaches such as measurement of physicochemical parameters in colonic luminal content, mucosa-adherent microbiota composition and activity, colon mucosa histo-morphological analysis, permeability tests, and expression of numerous factors involved in epithelial inflammation and/or repair were used.

Research results

Indications of epithelial repair were observed early, while inflammation was still active. However, colitis-induced luminal colonic environment alterations and microscopic abnormalities of colon mucosa persisted even though inflammation had been resolved.

Research conclusions

The longitudinal evolution study of the overlapping events that participated in epithelial repair revealed modulation factors (Il-15, Il-33, and Saa) that may prove to be potential therapeutic targets for mucosal healing enhancement.

Research perspectives

Since repairing processes were launched by mucosal inflammation, the interventional time window is an important parameter to take into account in clinical trials aiming to accelerate intestinal mucosal healing.

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ORIGINAL ARTICLE

Basic Study

Lingguizhugan decoction attenuates diet-induced obesity and hepatosteatosis *via* gut microbiota

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Abstract

BACKGROUND

Obesity is a major risk factor for a variety of diseases such as diabetes, nonalcoholic fatty liver disease, and cardiovascular diseases. Restricting energy intake, or caloric restriction (CR), can reduce body weight and improve metabolic parameters in overweight or obese patients. We previously found that Lingguizhugan decoction (LZD) in combination with CR can effectively lower plasma lipid levels in patients with metabolic syndrome. However, the mechanism underlying CR and LZD treatment is still unclear.

AIM

To investigate whether CR and LZD improve metabolic parameters by modulating gut microbiota.

METHODS

We extracted the water-soluble components out of raw materials and dried as LZD extracts. Eight-week old male C57BL/6 mice were treated with a 3-d treatment regime that included 24 h-fasting followed by gavage of LZD extracts for 2 consecutive days, followed by a normal diet (ND) *ad libitum* for 16 wk. To test the effects of gut microbiota on diet-induced obesity, 8-wk old male C57BL/6 mice received fecal microbiota transplantation (FMT) from CR and LZD-treated mice every 3 d and were fed with high-fat diet (HFD) *ad libitum* for 16 wk. Control mice received either saline gavage or FMT from ND-fed mice receiving saline gavage as mentioned above. Body weight was monitored bi-weekly. Food

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consumption of each cage hosting five mice was recorded weekly. To monitor blood glucose, total cholesterol, and total triglycerides, blood samples were collected *via* submandibular bleeding after 6 h fasting. Oxygen consumption rate was monitored with metabolic cages. Feces were collected, and fecal DNA was extracted. Profiles of gut microbiota were mapped by metagenomic sequencing.

RESULTS

We found that CR and LZD treatment significantly reduced the body weight of mice fed with ND (28.71 ± 0.29 *vs* 28.05 ± 0.15, $P < 0.05$), but did not affect plasma total cholesterol or total triglyceride levels. We then transplanted the fecal microbiota collected from CR and LZD-treated mice under ND feeding to HFD-fed mice. Intriguingly, transplanting the mice with fecal microbiota from CR and LZD-treated mice potently reduced body weight (44.95 ± 1.02 *vs* 40.53 ± 0.97, $P < 0.001$). FMT also reduced HFD-induced hepatosteatosis, in addition to improved glycemic control. Mechanistic studies found that FMT increased OCR of the mice and suppressed the expression and protein abundance of lipogenic genes in the liver. Metagenomic analysis revealed that HFD drastically altered the profile of gut microbiota, and FMT modified the profile of the gut microbiota.

CONCLUSION

Our study suggests that CR and LZD improve metabolic parameters by modulating gut microbiota.

Key words: Obesity; Diabetes; Lipid metabolism; Hepatosteatosis; Gut microbiota

Core tip: This study shows that caloric restriction (CR) together with Lingguizhugan decoction (LZD) only slightly reduce body weight and blood glucose levels of normal diet (ND)-fed mice. Yet, transplanting the fecal microbiota of these mice into high-fat diet (HFD)-fed mice potently attenuated diet-induced obesity, hepatic steatosis, and hyperglycemia. Moreover, we found that fecal microbiota transplantation increases oxygen consumption rate of the mice and suppresses hepatic lipid biosynthesis. Using metagenomic sequencing, we further discovered that CR and LZD treatment alters the profile of ND-fed mice, and fecal microbiota transplantation alters HFD-induced changes in gut microbiota. Taken together, our study highlights that CR and LZD treatment exerts its metabolic improving effects *via* modulating gut microbiota.

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INTRODUCTION

The prevalence of obesity has drastically increased in recent decades and has become a major health issue worldwide. Excessive body weight or obesity is a major risk factor for a variety of diseases, such as insulin resistance, type 2 diabetes, dyslipidemia, hypertension, non-alcoholic fatty liver disease, cardiovascular disease, and metabolic syndrome (MetS)[1-4]. However, there is no specific medical treatment for obesity, and patients are often treated for their metabolic complications such as hyperlipidemia, diabetes and hypertension. The most effective way to reduce body weight is perhaps adapting a more active lifestyle, that is, limiting food intake and increasing physical activity. Patients with severe obesity can be treated by gastric bypass surgery, but there are potential side effects of the operation, such as vitamin or iron deficiency, malnutrition, and hernia, limiting its application as a treatment for overweight or less obese patients[5]. Caloric restriction (CR) has recently been shown to improve metabolic parameters of obese patients with and without MetS[6,7]. Additionally, we have found that Lingguizhugan decoction (LZD), a traditional Chinese medicine, can be used to treat obesity and non-alcoholic fatty liver disease[8],

as it potently decreases plasma total triglycerides (TG) and total cholesterol (TC) levels and blood glucose levels in patients with MetS, on top of the reductions induced by CR[7]. However, the underlying mechanism is still unclear. In the past decade, the gut microbiota has been found to play an important role in the development of obesity and its related diseases[9-13]. Interestingly, metformin, a commonly used medicine to treat diabetes, has been found to improve glycemic control in type 2 diabetes patients *via* modulating the gut microbiota[14]. We thus wondered whether CR in combination with LZD exerted its effects by modulating gut microbiota.

To this end, we first explored if CR in combination with LZD affects energy and lipid metabolism in mice without any disturbance in metabolism, that is, mice fed a normal diet (ND). We found that combined treatment of CR + LZD reduced body weight and blood glucose levels of ND-fed mice. To further clarify whether such effects were mediated by microbiota, we transplanted the fecal microbiota from CR + LZD treated ND-fed mice to high fat diet (HFD)-fed mice and studied its effects on metabolic parameters. Interestingly, we found that fecal microbiota transplantation (FMT) protected mice from diet-induced obesity and hepatosteatosis and lowered plasma lipid level by promoting fatty acid (FA) oxidation and inhibiting hepatic lipid biosynthesis. Our study thus highlights a novel pharmacological mechanism of combined treatment with CR + LZD in treating obesity and MetS.

MATERIALS AND METHODS

Preparation of herbal extracts

LZD, which is a combination of 12 commonly used herbal medicines in China, has been widely used in clinical practice to treat patients with MetS[7]. To test the effects of LZD on mice, we extracted the water-soluble components out of the raw materials (Kangmei Pharmaceutical Co., Ltd). In short, to prepare the herbal extracts, 50.5 g Radix Codonopsis, 40.4 g Cortex Poria, 50.5 g Rhizoma Atractyldis Macrocephalae, 30.3 g Ramulus Cinnamomi, 101 g Radix Astragali, 101 g Rhizoma Dioscoreae, 20.2 g Pericarpium Citri Reticulatae, 30.3 g Rhizoma Pinelliae, 67.3 g Semen Coicis, 40.4 g Radix Morindae Officinalis, 40.4 g Epimedium Folium, and 20.2 g Radix Glycyrrhizae were briefly washed three times with sterilized ultrapure water, and then submerged into 2 L 100 °C sterilized ultrapure water and incubated for 1 h at 100 °C in a water bath. The supernatant was then filtered with a 300-mesh filter to remove any large debris, then centrifuged at 100× g for 30 min to remove any small debris. Prepared supernatant was then freeze-dried using a vacuum freezing-dryer (Christ Inc, Germany), and 122.8 g dried powder was obtained and stored at -80 °C until use. For medicine administration, dried LZD extracts were re-suspended with sterilized ultrapure water and dosed at 4.14 g/kg body weight. This dose is comparable to the dose used for human subjects described elsewhere[7].

Animals

C57BL/6 mice were purchased from Guangdong Medical Laboratory Animal Center (Guangzhou, China) and were housed at a 12-h light/12-h dark cycle. To test the effects of CR in combination with LZD extracts, hereby denoted as CR + LZD treatment, 8-wk old male C57BL/6 mice were treated with a 3-d treatment cycle, which includes 24 h-fasting and LZD extracts gavage for 2 consecutive days, and fed with ND (Research Diet, United States) ad libitum for 16 wk. Control mice did not undergo 24 h-fasting and were given saline gavage instead of LZD extracts gavage. To test the effects of gut microbiota on diet-induced obesity, 8-wk old male C57BL/6 mice received FMT from CR + LZD treated mice every 3 d and were fed with HFD (60% kcal fat, Research Diet) ad libitum for 16 wk. Control mice received either saline gavage or FMT from ND-fed mice receiving saline gavage as above-mentioned. Experimental schemes are summarized in Figure 1. Body weight was monitored bi-weekly. Food consumption of each cage hosting five mice was recorded weekly. To monitor blood glucose, TC, and triglycerides, blood samples were collected *via* submandibular bleeding after 6 h fasting. Experimental procedures were approved by the local animal ethnic committee (2018-057) and met the requirements of National Institutes of Health guide for the care and use of laboratory animals.

Feces collection and FMT

To collect feces, mice were housed individually in regular cages without any padding for 1-2 h, and feces were collected manually and chilled immediately with ice until use. For each group, 1 g feces was weighed, homogenized with 2.5 mL sterilized phosphate buffered saline using a micro-tube homogenizer, and filtered with a 40 μm

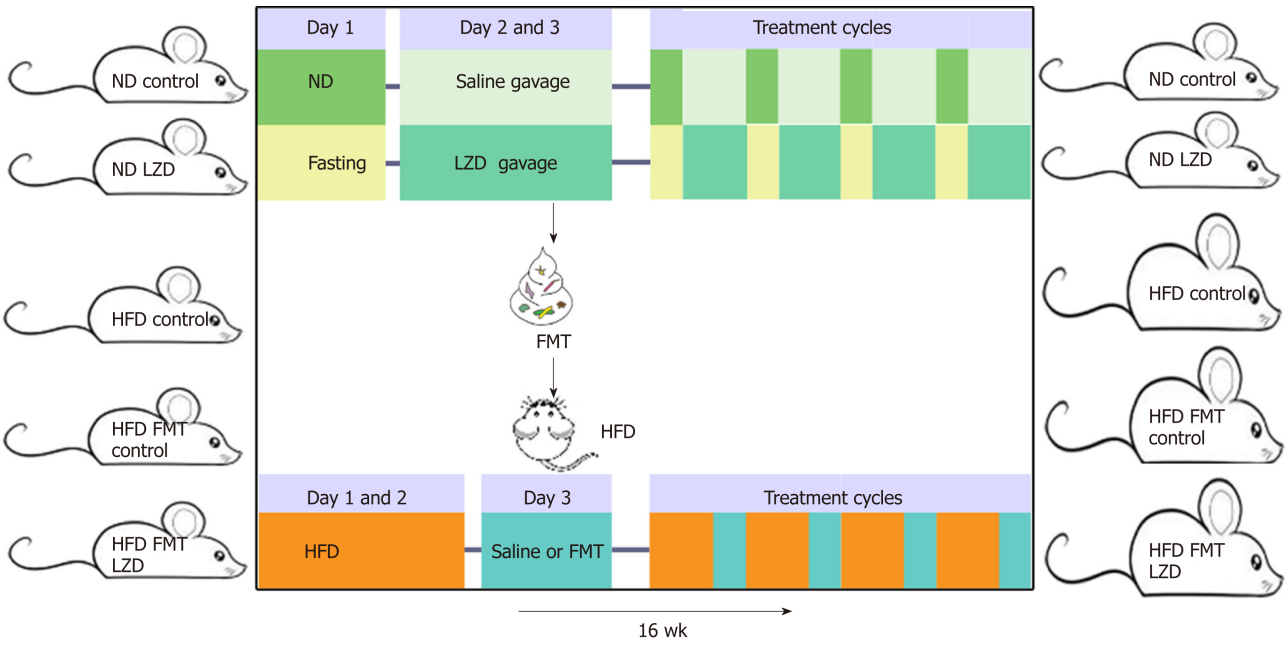

Figure 1 Illustration of experimental schemes. ND: Normal diet; LZD: Lingguizhigan decoction; HFD: High-fat diet; FMT: Fecal microbiota transplantation.

cell strainer (Thermo Scientific) to remove any large debris. Prepared fecal homogenates were stored on ice and used within 2 h. For FMT, 200 μL fecal homogenates were given to mice by oral gavage every 3 d.

Intraperitoneal glucose tolerance test

One week prior to the end of the study, an intraperitoneal glucose tolerance test was performed as previously described[15]. Briefly, after 16 h fasting, 2 g/kg glucose was injected intraperitoneally to mice. Blood samples were collected from tail vain at 0, 15, 30, 60 and 120 min after glucose injection, and blood glucose levels were measured using a glucometer (Johnson and Johnson). Glucose levels were compared at each time point, and overall differences in glycemic control were compared using area under the curve.

Oxygen consumption and physical activity measurement

Metabolic and physical activity of the mice were measured using an indirect open-circuit calorimeter (Oxylet, Panlab)[16]. In short, mice were housed individually in metabolic chambers and O_2 consumption, CO_2 production, and physical activity were recorded at 30 min intervals for 60 consecutive hours. For accuracy, the recorded data from the first 12 h after the mice have been housed in the chamber were not analyzed. Respiratory quotient (RQ) was calculated as the ratio between CO_2 production and O_2 consumption.

Hematoxylin and eosin staining and oil red O staining

Hematoxylin and eosin (H and E) and oil red O (ORO) staining were performed as described previously (Ref). In short, H and E was performed on 5 μm liver sections that were Bouin's-fixed and paraffin-embedded. For ORO staining, which was used to detected neutral lipids, fresh liver tissues embedded in optimal-cutting-temperature compound were cryosectioned for 7 μm in thickness. After fixation with 4% paraformaldehyde, sections were stained with 0.3% ORO following standard procedures and counterstained with hematoxylin. Images were scanned using Cytation 5 Cell Imaging Multi-mode reader (BioTek Instruments, United States).

Measurement of plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrate (BUN), and insulin levels

To measure plasma levels of ALT, AST, BUN, and insulin, blood samples were collected after 6-h fasting, at the end of the study, and pre-cleared by centrifugation at 3000× g for 5 min. Insulin levels were measured with an ELISA kit from ALPCO (catalog nr. 80-INSMSU-E01) according to the manufacturer's protocol. Plasma ALT and AST activities and BUN levels were measured using a colorimetric kit from Biosino (China), following the manufacturer's standard protocol.

RNA isolation and qPCR analysis

Liver samples were homogenized with Trizol (Thermo Fisher Scientific), and total RNA was extracted using Direct-zol RNA Miniprep kit (ZYMO Research). One microgram of total RNA was reverse transcribed with PrimeScript™ RT Master Mix (TaKaRa). SYBR Green real-time quantitative PCR assays were performed on a qTOWER apparatus (Analytic Jena) using SYBR® Premix Ex *Taq*™ II kit (TaKaRa). The expression of sterol regulatory element-binding protein (SREBP)-1c, acetyl-CoA carboxylase (ACC) α, fatty acid synthase (FASN), stearoyl-CoA desaturase (SCD) 1, and peroxisome proliferator-activated receptor (PPAR) γ were measured. SREBP1-c is a membrane-bound transcription factor that plays an important role in regulating lipogenic gene expression[17]. PPARγ plays an important role in HFD-induced upregulation of lipogenic gene expression and hepatic lipid biosynthesis[18,19]. ACC catalyzes acetyl-CoA to form malonyl-CoA, an essential substrate for synthesizing FA and an inhibitor of FA oxidation[20]. Thus, ACC levels are crucial in determining lipid metabolism and overall energy metabolism[21]. FASN is a large multipeptide enzyme whose major function is synthesizing palmitate, a long-chain saturated FA[22]. SCD1 is a key enzyme in catalyzing the synthesis of unsaturated FA oleic acid. Primers used in the study were as follow: ACCα (Forward: 5′-atgggcggaatggtctctttc-3′; Reverse: 5′-tggggaccttgtcttcatcat-3′), PPARγ (Forward: 5′-gaaagacaacggacaaatcacc-3′; Reverse: 5′-gggggtgatatgtttgaacttg-3′), SCD1 (Forward: 5′ttccctcctgcaagctctac-3′; Reverse: 5′-cagagcgctggtcatgtagt-3′), SREBP-1c (Forward: 5′-ggttttgaacgacatcgaaga-3′; Reverse: 5′-cgggaagtcactgtcttggt-3′), FASN (Forward: 5′-gctgctgttggaagtcagc-3′; Reverse: 5′-agtgttcgttcctcggagtg-3′), and 36B4 (Forward: 5′-actggtctaggacccgagaag-3′; Reverse: 5′-ctcccaccttgtctccagtc-3′).

Immunoblotting

For immunoblotting, 50 mg mouse liver samples were homogenized in RIPA buffer (Beyotime, China) with a protease inhibitor cocktail (Roche) using a TissueLyzer (Jingxin, China). Homogenates were precleared by centrifugation at 10000× g for 10 min at 4 °C. Protein concentrations were determined using BCA assay (Pierce). Equal amounts of proteins (20-30 mg) were loaded and separated on 4%-12% Bis-Tris gels (GenScript) and transferred to PVDF membranes using iBlot® 2 Dry Blotting System (Thermo Fisher Scientific). The blots were then probed with the following antibodies: Anti-ACC (1:1000, Cell Signaling Technology), anti-PPARγ (1:1000, Proteintech), anti-SCD1 (1:500, Abcam), anti-SREBP-1c (1:1000, Proteintech), anti-FASN (1:1000, Proteintech), anti-β-actin (1:5000, Proteintech), anti-mouse IgG (1:5000, Jackson ImmunoResearch), anti-rabbit IgG (1:5000, Jacson ImmunoResearch), and then detected by Clarity™ Western Substrate (Bio-Rad). Band intensities were analyzed using ImageJ.

Hepatic lipid extraction and measurement

To measure lipid contents in the liver, Folch's method was used to extract hepatic lipids[23]. In short, 50 mg liver samples were homogenized using TissueLyzer (60 Hz, 30 s) in chloroform and methanol mixture (2:1) followed by adding methanol, chloroform and ultrapure water. Extracted lipids were dried with N_2 gas and dissolved with 200 μL PBS containing 1% Triton X-100. Cholesterol and triglycerides were measured using colorimetric kits (Wako).

Metagenomic sequencing and analysis

Metagenomic sequencing and analysis were performed by Novagen (Beijing, China). In short, 40 mg feces from each mouse were pooled, and DNA was extracted using an automated DNA extractor (Chemagic360, PelkinElmer). DNA concentration was measured using Qubit® dsDNA Assay Kit in Qubit® 2.0 Fluorometer (Life Technologies, CA, United States), and DNA quality was checked using Labchip GX Touch 24 and HT DNA extended range Labchip kit. For library construction, 1 μg DNA per sample was used as input material. Sequencing libraries were generated using NEBNext® Ultra™ DNA Library Prep Kit for Illumina (NEB, United States) following the manufacturer's recommendations. Index codes were added to attribute sequences to each sample. Briefly, the DNA sample was fragmented by sonication to a size of 350 bp, then DNA fragments were end-polished, A-tailed, and ligated with the full-length adaptor for Illumina sequencing with further PCR amplification. At last, PCR products were purified (AMPure XP system), and libraries were analyzed for size distribution by Agilent2100 Bioanalyzer and quantified using real-time PCR.

For data processing and analysis, raw data obtained from the Illumina HiSeq sequencing platform were first preprocessed using Readfq (version 8, ht tps://github.com/cjfields/readfq) to acquire clean data for downstream analysis. The criteria for processing the raw data were as follows: (1) Reads that contain low quality bases (default quality threshold value ≤ 38) above a certain portion (default length of

40 bp) were removed; (2) Reads in which the N base has reached a certain percentage (default length of 10 bp) were removed; (3) Reads that shared an overlap above a certain portion with adapter (default length of 15 bp) were removed. Obtained clean data were further blasted to the host (mouse) database using Bowtie (version 2.2.4, http://bowtiebio.sourceforge.net/bowtie2/index.shtml) to remove any genes that were of host origin. To assemble metagenomes from the acquired clean data, SOAPdenovo software (version 2.04, http://soap.genomics.org.cn/soapdenovo.html) was used. For gene prediction and abundance analysis, MetaGeneMark (version 2.10, http://topaz.gatech.edu/GeneMark) and CD-HIT (version 4.5.8, http://www.bioinformatics.org/cd-hit) were used. For taxonomy prediction, DIAMOND (version 0.9.9, https://github.com/bbuchfink/diamond), NR database (version 2018-01-02, https://www.ncbi.nlm.nih.gov), MEGAN software, R vegan package (version 2.15.3), and LEfSe software were used. Detailed parameters used in the analysis are available upon request.

Statistics

All values were presented as mean ± SE. For experiments with n number ≥ 8/group, a D'Agostino-Pearson omnibus test was performed to test normality. For experiments with a lower n number, a Kolmogorov-Smirnov test was performed for normality test. After passing the normality test, one-way ANOVA followed by the Bonferroni test was performed for comparison of more than two groups. A student's t-test was used to compare the differences between two groups. P values of < 0.05 were considered significant. The statistical methods of this study were reviewed by Dr. Huachun Zhou from the Department of Medical Statistics and Epidemiology, School of Public Health, Sun Yat-Sen University.

RESULTS

CR and LZD reduces body weight of ND-fed mice

We first tested the effect of CR + LZD on ND-fed mice. To limit energy intake without causing any detrimental effect, the mice were fasted for 24 h every 3 d, and they received LZD by oral gavage. This treatment, in line with previous reports, reduced body weight (Figure 2A) without affecting accumulated food intake during the 16-wk experimental period (Figure 2B). It is likely that fasted mice consumed more food after fasting, thus leveling up the total food intake. Likely as a consequence of reduced body weight, blood glucose levels were also reduced approximately 20% by CR + LZD treatment, as compared to the levels of control treated mice (Figure 2C). Plasma levels of TG and TC were not affected by the treatment (Figure 2D and E), likely because these mice had normal baseline plasma lipid levels. To exclude the possibility that reduced body weight is not a result of liver injury induced by CR + LZD treatment, we measured plasma ALT and AST activities of the mice and found no changes in their activities and ratios (Figure 2F-H). This suggests that CR + LZD treatment did not impair liver functions. Additionally, we found that plasma levels of blood urea nitrogen, an indicator of renal damage, was unaltered by the treatment (Figure 2I), suggesting that the treatment did not cause renal damage. Taken together, these findings suggest that CR + LZD treatment is also effective in modulating metabolism in mice without any metabolic disorders.

FMT attenuates diet-induced obesity and hepatosteatosis in C57BL/6 mice

We speculated whether FMT from CR + LZD-treated mice would provide beneficial effects against diet-induced obesity in C57BL/6 mice. We collected feces from ND-fed mice receiving control treatment or CR + LZD treatment and transplanted the gut microbiota by oral gavage of fecal homogenates. Intriguingly, FMT from CR + LZD-treated mice attenuated HFD-induced body weight gain in C57BL/6 mice (Figure 3A). In accordance with reduced body weight, HFD-induced increase in blood glucose levels was also attenuated by FMT from CR + LZD-treated mice (Figure 3B). However, plasma insulin levels remained unaltered (Figure 3C), suggesting that treatment improved insulin sensitivity. Glucose tolerance was also improved by FMT from CR + LZD-treated mice, but not from control mice (Figure 3D). Similar to the effects in ND-fed mice, FMT from CR + LZD-treated mice lowered plasma TG and TC levels (Figure 3E and F). However, reduced plasma lipid levels could be caused by reduced hepatic lipid output, which could in turn cause lipid accumulation in the liver. To exclude this possibility, we examined hepatic lipid content by ORO staining. Strikingly, hepatic lipid staining was also reduced by FMT (Figure 3G). We further extracted lipid contents from the liver. When we measured lipid levels, we found that in line with ORO staining, hepatic TG and TC levels were both reduced in mice that

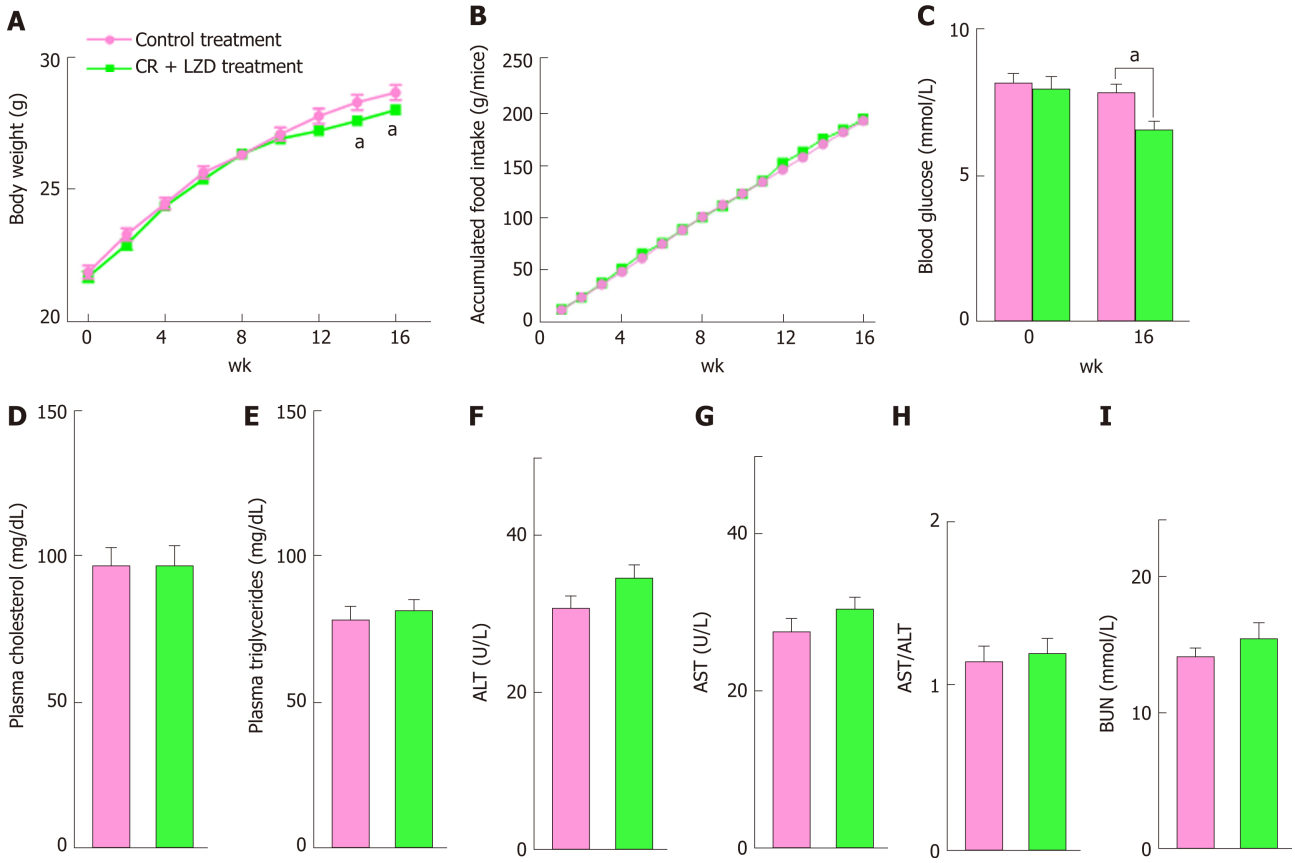

Figure 2 Caloric restriction and Lingguizhugan decoction treatment reduces body weight and blood glucose levels in normal diet-fed C57BL/6J mice. Eight-week-old male C57BL/6J mice received control treatment (pink) or caloric restriction and Lingguizhugan decoction (CR + LZD) (green) for 16 wk; *n* = 10 per group. A: Body weight was monitored biweekly during the study period, and each point and error represents the mean ± standard error; B: Accumulated food intake during experimental period; C: Fasting blood glucose levels were measured on weeks 0 and 16; D: Plasma total cholesterol levels; E: Plasma total triglyceride levels; F-H: Plasma alanine transaminase (ALT) and aspartate transaminase (AST) activity, and AST/ALT ratio; I: Plasma blood urea nitrogen levels. Data were evaluated for statistical significance by student's t-test and are represented as follows: $^{a}P < 0.05$ CR + LZD treatment *vs* Control treatment. CR: Caloric restriction; LZD: Lingguizhugan decoction; AST: Aspartate transaminase; ALT: Alanine transaminase; BUN: Blood urea nitrogen.

received FMT from CR + LZD-treated ND-fed mice (Figure 3H and I). These findings suggest that FMT either promoted lipid oxidation or inhibited lipid biosynthesis to reduce plasma and hepatic lipid levels. To gain a better understanding, we measured oxygen consumption rate (OCR) and physical activity of the mice. FMT from mice treated with CR + LZD, but not from control mice, drastically increased OCR (Figure 3J and K) without affecting physical activity (Figure 3L), suggesting that these mice were more active in metabolism. Additionally, RQ, a reflector for fuels being oxidized, was reduced by FMT (Figure 3M), implying that FA oxidation is likely increased. Taken together, these findings imply that FMT from CR + LZD-treated mice promotes lipid oxidation, thus reducing body weight and hepatic lipid contents.

FMT suppresses lipid biosynthesis in the liver

It is unclear whether FMT reduced hepatic lipid levels solely by increasing lipid oxidation. Thus, we measured the hepatic expression of key genes involved in lipid biosynthesis. We found that HFD increased expression of SREBP-1c, ACCα, FASN, SCD1, and PPARγ in the liver[24], as compared to the expression levels in the liver of ND-fed mice (Figure 4A-E). Thus, increased expression of these lipogenic genes explains the increases in lipid deposition in the liver and adipose tissues. Intriguingly, FMT from CR + LZD-treated mice, but not from control mice, reduced hepatic expression of these lipogenic genes (Figure 4A-E). Furthermore, we measured protein abundance of these key factors by immunoblotting. In line with gene expression profiles, SREBP-1c, ACCα, FASN, SCD-1 and PPARγ proteins were all reduced by FMT (Figure 4F and G). Taken together, these data suggest that FMT also suppresses lipid biosynthesis in the liver, further explaining the observation that hepatic lipid content was reduced by FMT.

FMT alters the profile of gut microbiota

To gain a better understanding of FMT-induced changes in metabolic profiles, we

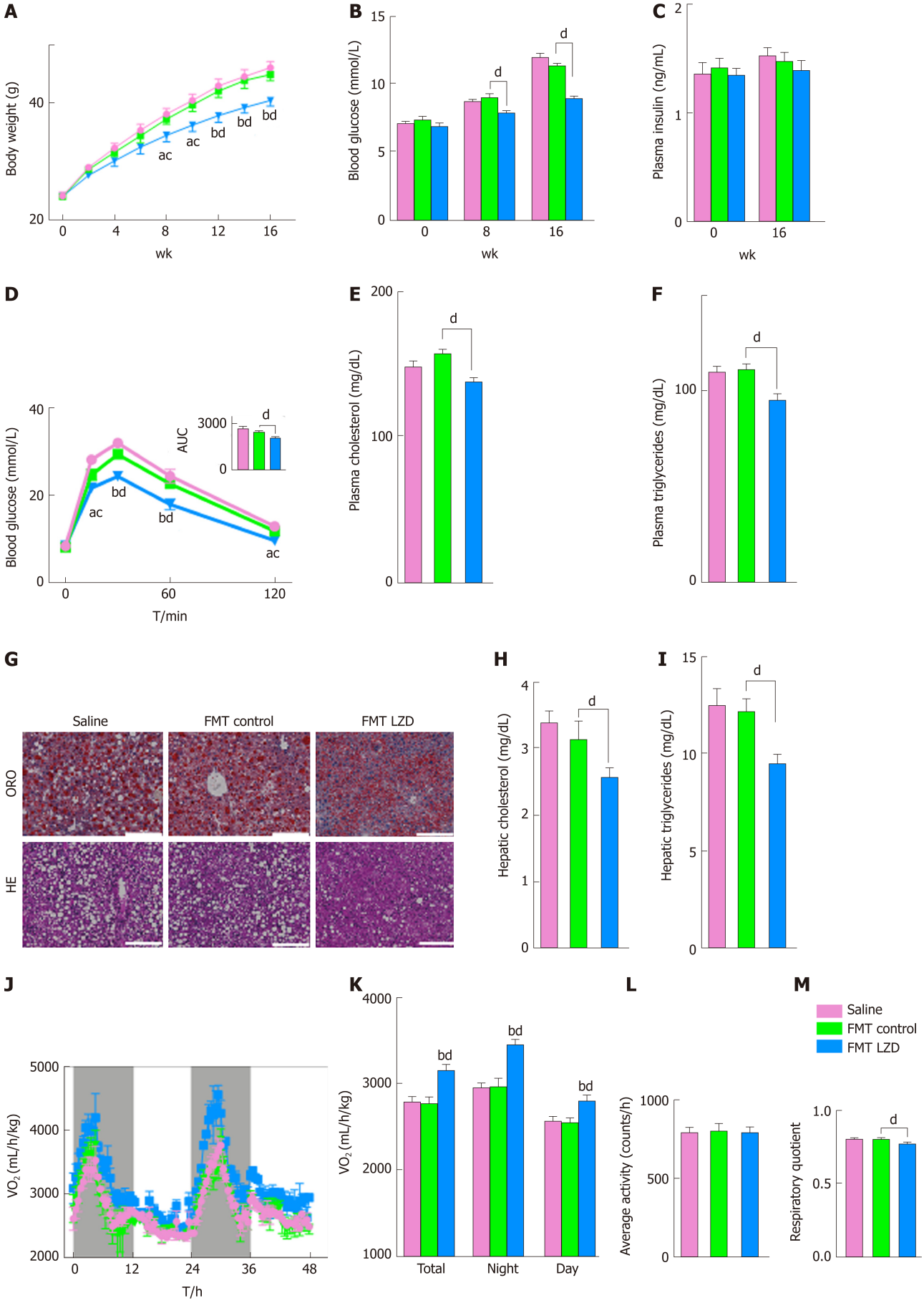

Figure 3 Fecal microbiota transplantation from caloric restriction and Lingguizhugan decoction-treated normal diet-fed mice into high-fat diet-fed C57BL/6J mice attenuates diet-induced obesity and hepatosteatosis. Eight-week-old male C57BL/6J mice received saline gavage (denoted as Saline, pink), feces gavage from normal diet (ND)-fed control mice [denoted as fecal microbiota transplantation (FMT) control, green], or feces gavage from ND-fed mice receiving

caloric restriction and Lingguizhugan decoction (CR + LZD) treatment (denoted as FMT LZD, blue) for 16 wk, and fed with HFD; *n* = 10 per group. A: Body weight was monitored biweekly during the experimental period, and each point and error represents the mean ± standard error; B: Fasting blood glucose levels were measured on weeks 0, 8 and 16; C: Plasma insulin levels were measured on weeks 0 and 16; D: Intraperitoneal glucose tolerance test was performed 1 wk prior to the end of the study. Glucose levels at each time point were compared by one-way ANOVA, and small letters indicate significant differences between FMT control and FMT LZD. Area under the curve was used to compare the overall differences between groups; E and F: Plasma total cholesterol (TC) and total triglycerides (TG) levels, respectively; G: Representative images of oil red O staining and hematoxylin and eosin staining of the liver (scale bar = 200 μm); H: Total cholesterol levels; I: Triglyceride levels; FMT control *vs* FMT LZD; J: Oxygen consumption of total test period, during the night, and during the day; K: Average oxygen consumption of total test period, during the night, and during the day; L: 24 h average physical activity; M: Respiratory quotient and of saline-treated, FMT control, and FMT LZD-treated mice were monitored with a metabolic monitoring system 3 d prior to death; *n* = 3 per group. Data were evaluated for statistical significance by one-way ANOVA and are represented as follows: $^{a}P < 0.05$, $^{b}P < 0.01$ FMT LZD *vs* Saline; $^{c}P < 0.05$, $^{d}P < 0.01$ FMT LZD *vs* FMT control. ND: Normal diet; LZD: Lingguizhigan decoction; HFD: High-fat diet; FMT: Fecal microbiota transplantation; AUC: Area under the curve; TC: Cholesterol; TG: Triglycerides; OCR: Oxygen consumption rate; ORO: Oil red O staining; HE: Hematoxylin and eosin staining.

mapped the gut microbiota by metagenome sequencing. Gut microbiota profile of the mice fed an ND was completely different from that of mice fed an HFD (Figures 5-8). At the phylum level, *Fibrobacteres, Spirochaetes, Fusobacteria, Firmicutes, Actinobacteria, Chlorobi, Candidatus, Cloacimonets, Elusimicrobia*, and *Candidatus Shapirobacteria* were abundantly detected in the feces of ND-fed mice, while they were less abundantly detected in the feces of HFD-fed mice (Figure 5). FMT from CR + LZD-treated mice into HFD-fed mice increased the abundance of the phyla *Fusobacteria, Actinobacteria, Elusimicrobia* and *Candidatus Shapirobacteria*, suggesting that alterations in gut microbiota induced by HFD were partially reversed by FMT. Additionally, the abundance of the phyla *Thermotogae, Chlamydiae, Aquificae, Candidatus Melainabacteria, Euryarchaeota, Tenericutes, Candidatus Yanofskybacteria*, and *Candidatus Nealsonbacteria* was increased by FMT from CR + LZD-treated mice (Figure 5). At the family level, FMT from CR + LZD-treated mice into HFD-fed mice increased the abundance of *Siphoviridae, Rikenellaceae, Odoribacteraceae, Oscillospiraceae, Chlamydiaceae, Clostridiales Family XIII, Incertae Sedis, Clostridiceae, Tannerellaceae* and *Bcillaceae* compared to ND-fed control mice and HFD-fed mice receiving saline gavage (Figure 6). At the genus level, FMT from CR + LZD-treated mice into HFD-fed mice strongly increased the abundance of *Rumiococcus*, *Proteus*, *Parabacteroides*, *Oscillibacter*, *Phascolarctobacterium*, *Alistipes*, *Desulfovibrio*, and *Clostridium* (Figure 7). At the species level, FMT from CR + LZD-treated mice into HFD-fed mice increased the abundance of *Alistipes finegoldii*, *Alistipes puredinis*, *Bacteroides coprophilus*, *Clostridium* sp. CAG: 389, *Clostridium* sp. CAG: 343, *Phascolarctobacterium* sp. CAG: 207, and uncultured *Ruminococcus sp.* (Figure 8).

DISCUSSION

Regular or prolonged CR is effective in treating metabolic disorders, such as obesity, diabetes, hypertension and hyperlipidemia[25,26]. CR + LZD treatment can effectively lower plasma TC and TG levels in patients with MetS[7]. Similarly, CR + LZD attenuates HFD-induced obesity and hyperlipidemia in rats[27]. Despite its effectiveness, little is known about its underlying mechanism. In the current study, we demonstrated that CR + LZD treatment is also capable of lowering blood glucose levels and body weight in ND-fed C57BL/6 mice. Yet, it is not clear how LZD reduces body weight. Perhaps, as illustrated in HFD-fed mice receiving FMT of CR + LZD treated mice, CR + LZD may increase OCR of the mice by promoting FA oxidation. However, unlike previous findings, plasma TC and TG levels were not affected by the treatment. It is possible that CR + LZD treatment only attenuates dyslipidemia induced by excessive fat intake because under ND feeding, C57BL/6 mice have normal plasma lipid levels. This implies that CR + LZD treatment is more likely correcting disturbed lipid metabolism rather than directly modulating plasma lipid metabolism. We found that the gut microbiota profile of C57BL/6 mice is drastically changed by HFD feeding. Recent studies have highlighted the importance of gut microbiota in the development of metabolic diseases, such as obesity and diabetes. It is thus possible that CR + LZD can modulate gut microbiota to improve metabolism. Indeed, we found that FMT from CR + LZD-treated mice attenuated HFD-induced obesity, hyperglycemia, and hepatosteatosis. FMT improves metabolic parameters to a similar degree as those achieved by CR + LZD treatment in HFD-fed rats[27], highlighting that the gut microbiota is the primary, if not the only, target of CR and LZD in modulating metabolism. Recent studies have also revealed that traditionally used herbal medicines, such as *Ganoderma lucidum* and *Hirsutella sinensis*, exert their effects by modulating gut microbiota[28,29]. These findings, including ours, present a new aspect in understanding the pharmacological effects of herbal plants. Yet, it is

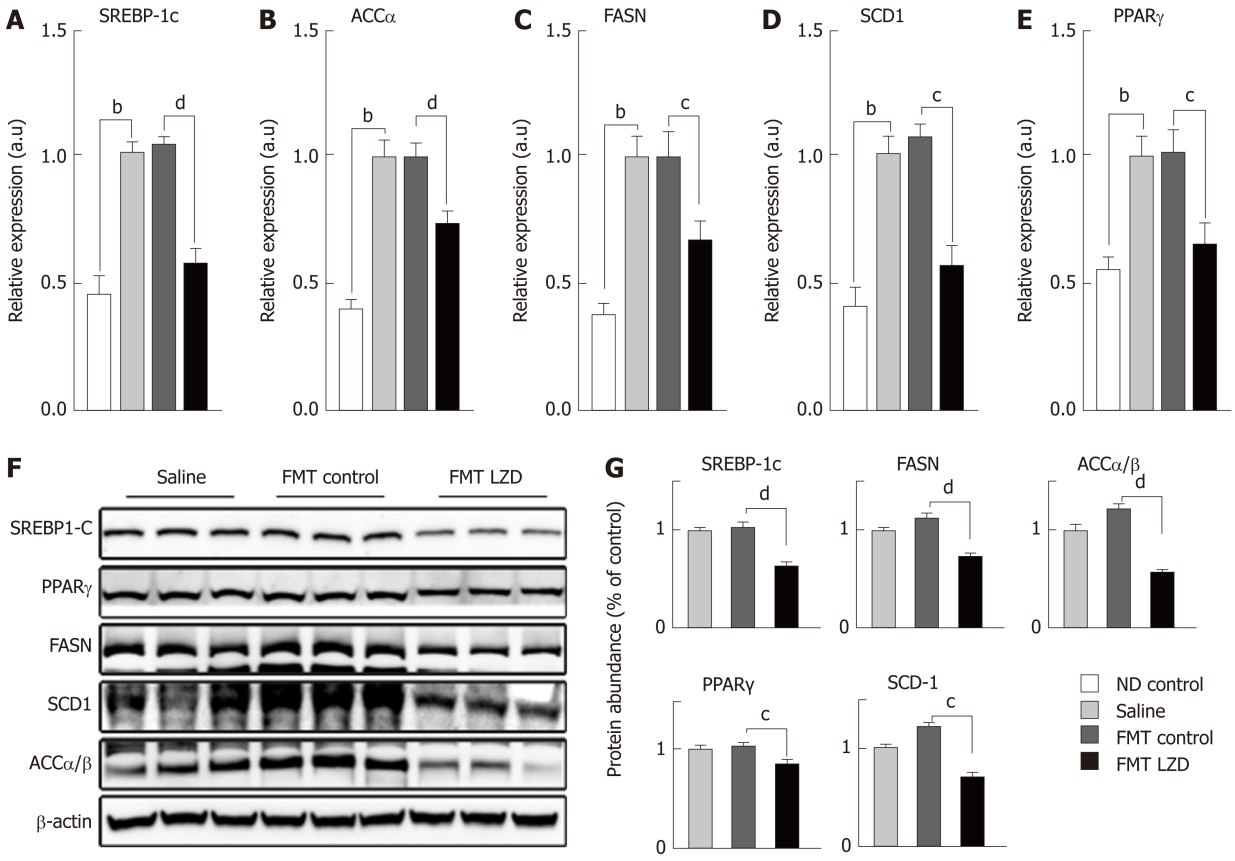

Figure 4 Fecal microbiota transplantation from caloric restriction and Lingguizhugan decoction-treated normal diet-fed mice into high-fat diet-fed C57BL/6J mice reduces expression level and protein abundance of lipogenic genes. Total RNA was isolated from liver samples from saline-treated mice (pink), fecal microbiota transplantation (FMT) control mice (green), and FMT Lingguizhigan decoction (LZD)-treated mice (blue). A-E: Gene expression was analyzed and normalized to 36B4 expression in the same sample; *n* = 10 per group; F: Representative blot of liver samples; G: Protein abundance of sterol regulatory element-binding protein 1c, peroxisome proliferator-activated receptor γ, fatty acid synthase, stearoyl-CoA desaturase 1, and acetyl-CoA carboxylase α/β was quantified and normalized to β-actin levels in the same lysate; *n* = 9 per group. Data were evaluated for statistical significance by one-way ANOVA and are represented as follows: $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* normal diet control with Saline; $^{c}P < 0.05$, $^{d}P < 0.01$ FMT LZD *vs* FMT control. ND: Normal diet; LZD: Lingguizhigan decoction; HFD: High-fat diet; FMT: Fecal microbiota transplantation; SREBP: Sterol regulatory element-binding protein; ACC: Acetyl-CoA carboxylase; FASN: Fatty acid synthase; SCD: Stearoyl-CoA desaturase; PPAR: Peroxisome proliferator-activated receptor.

still not clear how these herbal medicines affect the composition of gut microbiota. It is possible that components of the medical plants inhibit the growth of certain bacteria while promoting the growth of others. Extract of *Glycyrrhiza glabra* has been shown to inhibit the growth of *Salmonella*, *Shigella* and enterotoxigenic *Escherichia coli in vivo*[30]. It is worth noting that *Radix Glycyrrhizae*, a component of LZD, contains dried roots and rhizomes of *Glycyrrhiza glabra*. Another possibility is that the components of the medical plants can be transformed into other metabolites that facilitate the growth of certain types of microbes or directly affect the metabolism of the host, as reviewed elsewhere[31,32].

It must be noted that the gut microbiota profile of CR + LZD-treated and ND-fed mice did not overlap with that of HFD-fed mice that received FMT. The exact reason for this is not clear. Possibly, the most abundant microbes present in the donor feces did not necessarily well inhabit the gut of the recipient mice. Recent studies have reported that HFD increases the abundance of the phyla Firmicutes and Proteobacteria and decreases the abundance of *Bacteroidetes*[33,34]. In the current study, we found that HFD decreased the abundance of phyla *Firmicutes* and *Bacteroidetes*, but increased the abundance of *Proteobacteria*, differing with previous report. It is possible that the origin of the mice and the housing environment may have had an impact on the gut microbiota profile. Yet, we found that FMT from CR + LZD-treated mice increased the abundance of species *Alistipes finegoldii*, *Alistipes putredinis* and *Bacteroides coprophilus*, which belong to the phyla *Bacteroidetes*. Of note, a decreased abundance of *Bacteroides coprophilus* has been reported in MetS patients[35]. *Alistipes putredinis* has recently been identified as a butyrate producer in the gut[36]. Butyrate is a short-chain FA and plays an important role in maintaining colon health. The main butyrate-producing bacteria are within the *Firmicutes* phylum, but members of the *Actinobacteria, Bacteroidetes, Fusobacteria, Proteobacteria, Spirochaetes* and *Thermotogae*

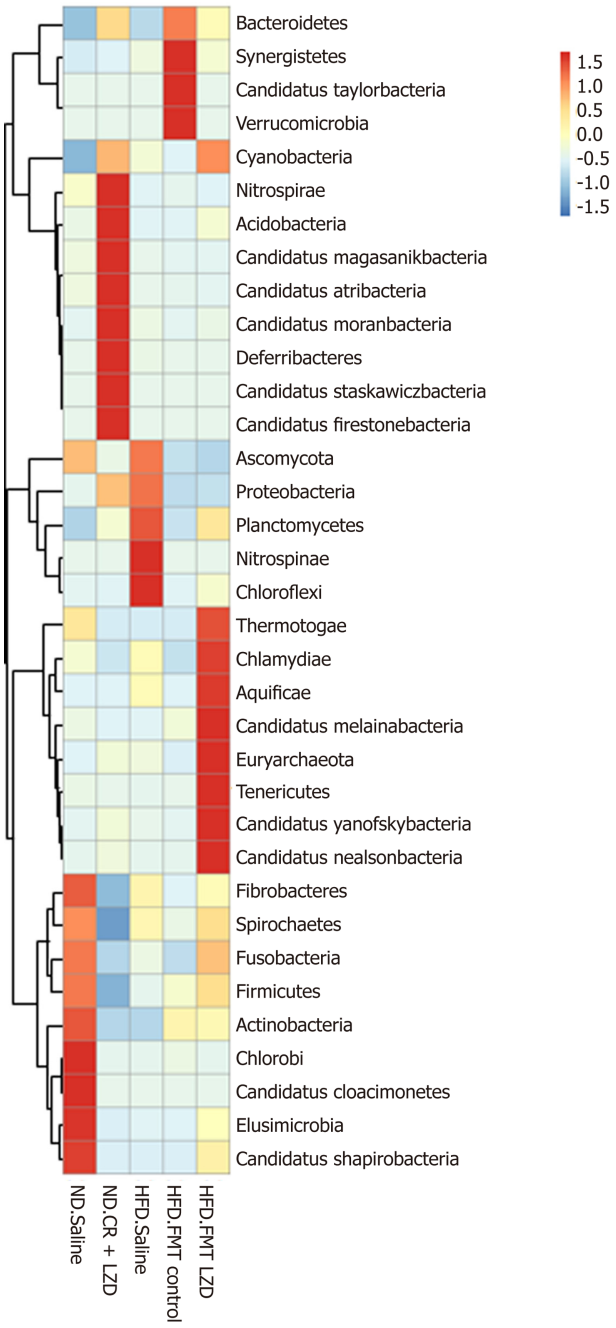

Figure 5 Relative abundance of gut microbes at phylum level. ND. Saline: Mice fed with normal diet (ND) and given saline gavage; ND. CR + LZD: Mice fed with ND on caloric restriction (CR) and Lingguizhigan decoction (LZD) treatment; HFD. Saline: Mice fed high-fat diet (HFD) and given saline gavage; HFD. FMT control: Mice fed HFD and given fecal microbiota transplantation (FMT) from ND. Saline mice; HFD. FMT CR + LZD: Mice fed HFD and given FMT from ND. CR + LZD mice; ND: Normal diet; LZD: Lingguizhigan decoction; HFD: High-fat diet; FMT: Fecal microbiota transplantation; CR: Caloric restriction.

phyla also have butyrate-synthesis pathways[36]. It is worth noting that FMT from mice treated with CR + LZD strongly increased the abundance of *Thermotogae*. Dietary butyrate supplementation can attenuate diet-induced obesity, insulin resistance, and hyperlipidemia in mice by activating mitochondrial functions[37,38]. Additionally, butyrate is also capable of reducing lipid secretion and lipoprotein biosynthesis in hepatic cells[39]. Interestingly, butyrate also increases OCR of epithelial cells[40]. Taken together, FMT from CR + LZD-treated mice could increase FA oxidation and limit lipid biosynthesis by increasing the abundance of butyrate-producing bacteria in the gut. In conclusion, we report that CR in combination with LZD attenuates diet-induced obesity and hepatosteatosis by modulating the gut microbiota.

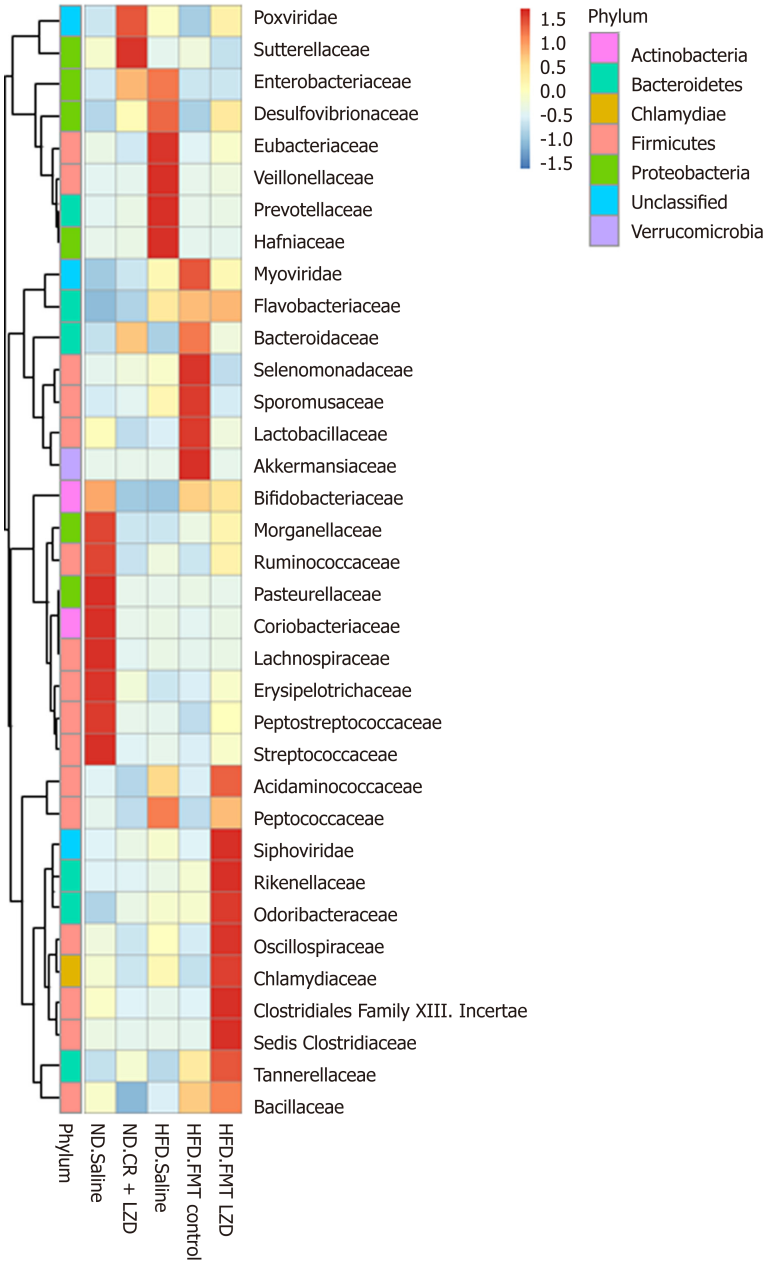

Figure 6 Relative abundance of gut microbiota at the family level. ND. Saline: Mice fed normal diet (ND) and given saline gavage; ND. CR + LZD: Mice fed ND and given caloric restriction (CR) and Lingguizhigan decoction (LZD) treatment; HFD. Saline: Mice fed high-fat diet (HFD) and given saline gavage; HFD. FMT control: Mice fed HFD and given fecal microbiota transplantation (FMT) from ND. Saline mice; HFD. FMT CR + LZD: Mice fed HFD and given FMT from ND. CR + LZD mice; ND: Normal diet; LZD: Lingguizhigan decoction; HFD: High-fat diet; FMT: Fecal microbiota transplantation; CR: Caloric restriction.

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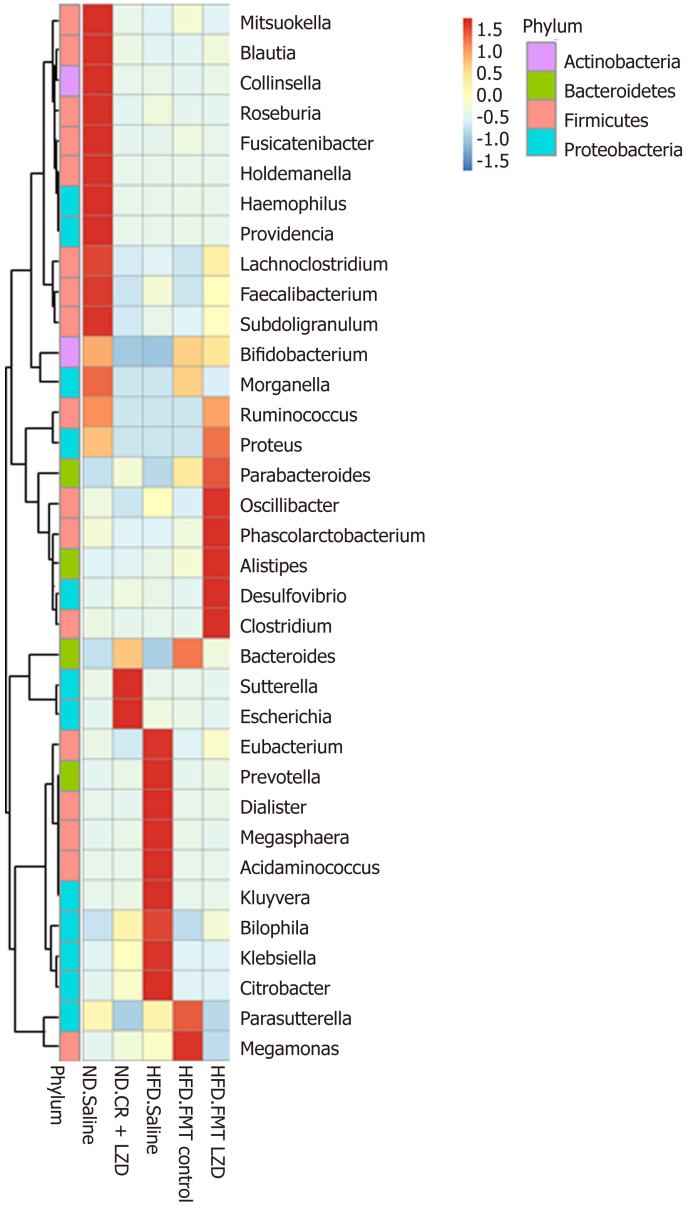

Figure 7 Relative abundance of gut microbiota at the genus level. ND. Saline: Mice fed normal diet (ND) and given saline gavage; ND. CR + LZD: Mice fed ND and given caloric restriction (CR) and Lingguizhigan decoction (LZD) treatment; HFD. Saline: Mice fed high-fat diet (HFD) and given saline gavage; HFD. FMT control: Mice fed HFD and given fecal microbiota transplantation (FMT) from ND. Saline mice; HFD. FMT CR + LZD: Mice fed HFD and given FMT from ND. CR + LZD mice; ND: Normal diet; LZD: Lingguizhigan decoction; HFD: High-fat diet; FMT: Fecal microbiota transplantation; CR: Caloric restriction.

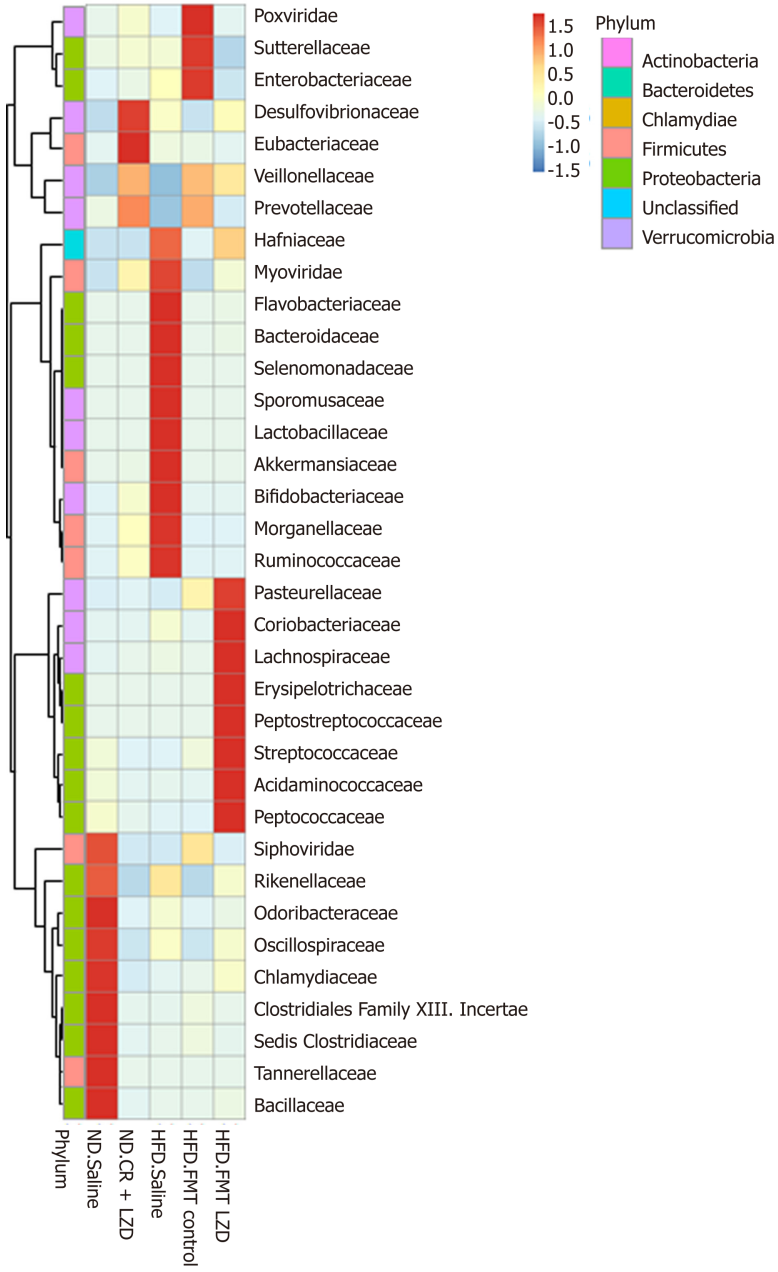

Figure 8 Relative abundance of gut microbiota at species level. ND. Saline: Mice fed a normal diet (ND) and given a saline gavage; ND. CR + LZD: Mice fed ND and given caloric restriction (CR) and Lingguizhigan decoction (LZD) treatment; HFD. Saline: Mice fed high-fat diet (HFD) and given saline gavage; HFD. FMT control: Mice fed HFD and given fecal microbiota transplantation (FMT) from ND. Saline mice; HFD. FMT CR + LZD: Mice fed HFD and given FMT from ND. CR + LZD mice; ND: Normal diet; LZD: Lingguizhigan decoction; HFD: High-fat diet; FMT: Fecal microbiota transplantation; CR: Caloric restriction.

ARTICLE HIGHLIGHTS

Research background

Lingguizhugan decoction (LZD) in combination with food restriction is widely used in clinical practice to treat patients with metabolic disorders, such as obesity, diabetes, high plasma lipid levels, and non-alcoholic fatty liver disease. Despite its wide application and effectiveness, little is known about its mechanism.

Research motivation

Although it is well accepted to use LZD in clinic to treat patients with metabolic disorders, lacking the knowledge on its mechanism has limited its use. Clarifying the pharmacological mechanism will provide scientific evidence for its clinic usage. Recent studies have revealed that traditional Chinese herbal medicines exert their effects by modulating gut microbiota, which has been previously unrecognized.

Research objectives

To investigate whether LZD combined with food restriction improves metabolic parameters *via*

modulating gut microbiota.

Research methods
To answer this question, we administered LZD gavage in addition to food restriction to mice fed a normal diet, and monitored body weight, blood glucose, and plasma lipid levels. At the same time, we collected the feces of these mice and homogenized with saline. We gave these fecal homogenates, which contain microbes, to mice fed a high fat diet. As high fat diet increases body weight, it causes increases in plasma lipid levels and blood glucose levels and induces abnormal lipid accumulation in the liver. Thus, we studied the effects of giving fecal homogenates from LZD treated and food-restricted mice on diet-induced metabolic abnormalities.

Research results
We found that LZD together with food restriction slightly reduced body weight and blood glucose levels but did not affect plasma lipid levels. However, giving the fecal homogenates collected from LZD and food-restricted mice greatly reduced body weight, plasma lipid levels, hepatic lipid contents, and blood glucose levels of mice on a high-fat-diet. We also found that giving the mice fecal homogenates significantly promoted fat oxidation and inhibited fat synthesis. Using DNA sequencing techniques, we found that LZD together with food restriction significantly changed the composition of bacteria in the gut.

Research conclusions
We found that a widely used traditional Chinese medicine can change the bacteria composition of the gut. Transferring these gut bacteria into high-fat-diet fed mice can reduce diet-induced increase in blood glucose, plasma lipid levels, hepatic lipid contents and body weight gain. Thus, gut microbes are the most likely primary target of LZD and food restriction treatment.

Research perspectives
Our study highlights the possibility of using bacteria to treat metabolic disorders such as obesity in the future. Using metagenomics, metatranscriptomic sequencing, and fecal metabolomics, it is possible to identify the most important bacteria and metabolites underlying the treatment of LZD and food restriction. This will make it possible to culture the identified bacteria *in vivo* and treat them with LZD extracts. Then giving the patients with such cultured and treated bacteria would provide similar effects as LZD treatment, thus reducing any potential toxic effects of the herbal medicine.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Intermediate-advanced hepatocellular carcinoma in Argentina: Treatment and survival analysis

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approval study protocol number 14-039) and from each Bioethics Institutional Committee from all participating centers.

Informed consent statement: All study participants from the prospective cohort, or their legal guardian, provided informed written consent prior to the study enrollment. From the retrospective cohort, all study investigators signed a confidential agreement. We submit the informed consent (IC) and it's Spanish version approved by the Austral University, School of Medicine and the Bioethics Institutional Committee of the Austral University Hospital (CIE approval study protocol number 14-039).

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Abstract

BACKGROUND

Hepatocellular carcinoma (HCC) represents the sixteenth most frequent cancer in Argentina. The rise of new therapeutic modalities in intermediate-advanced HCC opens up a new paradigm for the treatment of HCC.

AIM

To describe real-life treatments performed in patients with intermediate-advanced HCC before the approval of new systemic options.

METHODS

This longitudinal observational cohort study was conducted between 2009 and 2016 in 14 different regional hospitals from Argentina. Included subjects had intermediate-advanced Barcelona Clinic Liver Cancer (BCLC) HCC stages (BCLC B to D). Primary end point analyzed was survival, which was assessed for each BCLC stage from the date of treatment until last patient follow-up or death. Kaplan Meier survival curves and Cox regression analysis were performed, with hazard ratios (HR) calculations and 95% confidence intervals (95%CI).

RESULTS

From 327 HCC patients, 41% were BCLC stage B, 20% stage C and 39% stage D. Corresponding median survival were 15 mo (IQR 5-26 mo), 5 mo (IQR 2-13 mo) and 3 mo (IQR 1-13 mo) ($P < 0.0001$), respectively. Among BCLC-B patients ($n = 135$), 57% received TACE with a median number of 2 sessions (IQR 1-3 sessions). Survival was significantly better in BCLC-B patients treated with TACE HR = 0.29 (CI: 0.21-0.40) than those without TACE. After tumor reassessment by RECIST 1.1 criteria following the first TACE, patients with complete response achieved longer survival [HR = 0.15 (CI: 0.04-0.56, $P = 0.005$)]. Eighty-two patients were treated with sorafenib, mostly BCLC-B and C (87.8%). However, 12.2% were BCLC-D. Median survival with sorafenib was 4.5 mo (IQR 2.3-11.7 mo); which was lower among BCLC-D patients 3.2 mo (IQR 2.0-14.1 mo). A total of 36 BCLC-B patients presented tumor progression after TACE. In these patients, treatment with sorafenib presented better survival when compared to those patients who received sorafenib without prior TACE [HR = 0.26 (CI: 0.09-0.71); $P = 0.013$].

CONCLUSION

In this real setting, our results were lower than expected. This highlights unmet needs in Argentina, prior to the introduction of new treatments for HCC.

Key words: Hepatocellular carcinoma; Therapeutics; Survival; Real-life

Core tip: Trans-arterial chemoembolization and systemic treatment with sorafenib or lenvatinib are the standards of treatment for patients with intermediate and advanced stage hepatocellular carcinoma (HCC). The rise of new current therapeutic modalities such as radioembolization, the combination of antiangiogenic agents with locoregional therapies and other first and second line systemic options, open up a new paradigm for the treatment of HCC. In this dual cohort study, we describe the treatments performed in the real life setting before the approval of these new systemic options. Our real-data outcomes, lower than expected, highlight unmet needs and improvement areas in the daily practice prior to the introduction of new treatments for HCC.

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INTRODUCTION

According to the latest estimates made by the International Agency for Research on Cancer [IARC (http://gco.iarc.fr)] for the year 2018, Argentina has an incidence rate of 212 cases per 100000 inhabitants[1]. This figure places it within the countries of the world with medium-high incidence of cancer (range 177 to 245.6 per 100000 inhabitants) and in seventh place in Latin America. Although liver cancer or hepatocellular carcinoma (HCC) is currently the 5th most common cancer and the 2nd cause of death from cancer worldwide, in Argentina represents the sixteenth most frequent cancer (www.argentina.gob.ar/salud/instituto-nacional-del-cancer)[1].

Given that in more than 90% of the cases this tumor develops in patients with cirrhosis or chronic infection with hepatitis B or C virus, the diagnostic, staging and therapeutic management in our country is mainly done by hepatologists or hepatobiliary surgeons, rather than clinical oncologists[2].

Transarterial chemoembolization (TACE) and systemic treatment with sorafenib or lenvatinib are the standard treatments for patients with intermediate and advanced stage HCC[3-5]. The rise of new therapeutic modalities such as radioembolization, the combination of antiangiogenic agents with locoregional therapies and other first and second line systemic options, open up a new paradigm for the treatment of HCC.

In this dual cohort study, we aimed to describe treatments performed in the real life setting before the approval of these new systemic options. It is of interest to know the real life context, in order to evaluate the therapeutic management in these patients and gaps that should be explored more thoroughly as areas of public health improvement.

MATERIALS AND METHODS

Study design, setting and participating centers

This longitudinal observational cohort study was conducted in 14 different regional hospitals from Argentina. Two cohorts of consecutive adult patients (> 17 years of age) with newly diagnosed HCC were included. Between January 1 2009 and September 1 2014, a retrospective cohort was followed-up until death or last ambulatory visit until January 1 2016 (Cohort 1). A second prospective cohort was included from September 2 2014, followed until January 1 2016 (Cohort 2). Participating centers appointed a study coordinator responsible for data collection. Sites were instructed to enroll all eligible patients on a sequential basis and to record data from medical charts into a web-based electronic system. In cases of conflicting or missing data, central revision and resubmission was requested.

Cohort characteristics and study variables

Patients with intermediate (BCLC-B) or advanced-end stage (BCLC C-D) HCC were included[6,7]. Criteria for inclusion required patients to be adult recipients with newly diagnosed HCC either by pathological criteria or imaging evaluation as recommended by international Western guidelines[6,7]. Intermediate stage or BCLC B includes patients with preserved liver function with multifocal tumors, in the absence of cancer related symptoms, vascular invasion or extrahepatic spread. In these patients the recommended treatment is TACE. Advanced-stage HCC (BCLC C) comprises patients with preserved liver function, good performance status or ECOG 1-2, with extrahepatic spread (lymph node involvement or metastases) or vascular invasion. In this subgroup, sorafenib or lenvatinib are the recommended treatments. As lenvatinib has been recently approved in our country (March 2019), this cohort includes patients treated under sorafenib. Best supportive care (BSC) or symptomatic treatment is recommended for patients with unpreserved liver function (Child Pugh C) or ECOG > 2 or cancer related symptoms[8]. Patients were excluded if (1) clinical baseline data was missing; (2) BCLC stage was either 0 or A; and (3) patients with BCLC-B-D who underwent liver transplantation.

Baseline characteristics at HCC diagnosis included patients demographics, performance status (ECOG grade 0-4), grade of liver fibrosis (I-IV) assessed by liver biopsy or elastography or other non-invasive measurements or by clinical data (including imaging data, presence of gastro-esophageal varices or ascites or splenomegaly > 120 mm diameter, or other complications related to portal hypertension), Child Pugh score; selected laboratory variables, serum alpha-fetoprotein (AFP) levels and tumor characteristics at diagnosis, as well as treatments performed. Computed tomography (CT) or magnetic resonance images (MRI) were evaluated considering tumor number and diameter, vascular invasion and extrahepatic or lymph node metastasis.

Tumor treatment after HCC diagnosis was reviewed, namely: Liver resection (LR), radiofrequency ablation (RFA), percutaneous ethanol injection (PEI), trans-arterial chemoembolization (TACE), trans-arterial radioembolization (TARE), sorafenib and best supportive care (BSC). Each treatment was discussed at each center on a case-by-case basis. Imaging tumor reassessment after treatments were done according to RECIST 1.1 criteria as recommended by international Western guidelines[6,7].

Study end-points

Primary end point analyzed was survival, which was assessed for each BCLC stage from the date of treatment until last patient follow-up or death. Secondary objectives were to (1) describe treatments performed in each BCLC stage; (2) to evaluate the sequential treatment of TACE-sorafenib in BCLC-B patients; and (3) to evaluate adverse events and tolerability of sorafenib in the daily practice.

All procedures followed were in accordance with the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement[9]. This study was approved by the Austral University School of Medicine and by each center; complied with the ethical standards (institutional and national) and with Helsinki Declaration of 1975, as revised in 2008.

Statistical analysis

Statistical significance is expressed as $P < 0.05$. Categorical data were compared using Fisher's exact test or Chi-Square test. Continuous variables were compared with Student's *T* test or Mann-Whitney *U* test according to their distribution, respectively. Multiple comparisons for continuous data were done according to its distribution with ANOVA or Kruskal Wallis tests as appropriate. Dummies for ordinal variables were assessed. For survival analysis, Cox regression analysis estimating hazard ratios (HR) and 95%CI for baseline variables related with mortality was performed. Proportional hazards through graphic and statistical evaluation (Schoenfeld residual test) were done. Kaplan Meier survival curves were compared using the log-rank test (Mantel-Cox) Collected data was analyzed using STATA 13.0.

RESULTS

From a total of 721 consecutive adult patients with HCC during the study period, 327 patients with newly diagnosed intermediate and advanced HCC were included. Patients who received a liver transplant in BCLC-B (n = 16), BCLC-C (n = 2) and BCLC-D (n = 28) were excluded.

Table 1 describes the main baseline patient characteristics. Overall, 41.3% of the patients were in BCLC stage B (n = 135), 19.9% in stage C (n = 65) and 38.8% in stage D (n = 127). Treatments performed during the whole follow-up period were LR (n = 36), RFA or PEI (n = 19), TACE (n = 126), TARE (n = 6), sorafenib (n = 82) and BSC (n = 146).

Outcomes were assessed in all patients during follow-up with a median survival of 12.0 mo (IQR 4.0-27.0 mo). Corresponding median survival for BCLC stages were as follows: stage B 15 mo (IQR 5-26 mo), stage C 5 mo (IQR 2-13 mo) and stage D 3 mo (IQR 1-13 mo)(Figure 1).

Characteristics and management of patients treated with TACE

TACE was performed in 126 patients (38.5%); 77 were BCLC-B, 22 were BCLC-C and 27 patients were BCLC-D. According to the type of endovascular treatment, 43.6% of the patients were treated with conventional TACE (cTACE), 45.2% with TACE with drug eluting beads (TACE-DCbeads) and 11.2% with transarterial embolization (TAE).

Among BCLC-B patients (n = 135), 57% received TACE (n = 77) whereas 43% did not (Table 2). Median number of TACEs sessions was 2 (IQR 1-3 sessions); 40%, 26% and 34% of these patients received 1, 2 and 3 or more sessions, respectively. Other treatments than TACE were performed in BCLC-B patients, as follows: RFA or PEI in 7 patients, liver resection in 21 patients, sorafenib in 15 patients and BSC in 5 patients.

Of the 22 BCLC-C patients who were treated with TACE, 13 had non-main portal trunk vascular invasion and 12 patients had extrahepatic disease (lymph node metastasis in 5, bone metastasis in 3 and 4 patients with lung involvement). Sorafenib was the following treatment performed in 7 patients. Among BCLC-D, 27 patients received TACE, 19 were Child Pugh C, 10 patients presented performance status ECOG 3-4, 2 patients presented non-main portal trunk vascular invasion and 1 had extrahepatic disease (lymph node metastasis). Best supportive care following TACE was done in all patients except for 1 who received sorafenib in this latter group.

Survival was significantly better in BCLC-B patients treated with TACE HR 0.29

Table 1 Patients' baseline characteristics

Variable	Values
Age, yr (± SD)	63 ± 10
Male gender, *n* (%)	265 (81.3)
Non-cirrhotic liver, *n* (%)	41 (12.5)
Child Pugh A/B/C, *n* (%)	137 (42)/98 (30)/92 (28)
Etiology of liver disease, *n* (%)	
Hepatitis C virus	99 (30.3)
Alcohol	77 (23.5)
NASH	35 (10.7)
Cryptogenic	37 (11.3)
Hepatitis B virus	22 (6.7)
Cholestatic[1]	4 (1.2)
Autoimmune	-
Hemochromatosis	6 (1.8)
Miscellaneous	36 (11.0)
Comorbidities, *n* (%)	141 (43.1)
Diabetes mellitus, *n* (%)	84 (25.7)
Ascites, *n* (%)	
Mild	76 (23.3)
Moderate-severe	77 (23.5)
Encephalopathy, *n* (%)	
Grade I-II	78 (23.8)
Grade III-IV	6 (1.8)
CSPH, *n* (%)	212 (64.8)
ECOG 0-2, *n* (%)	262 (80.1)

[1]Cholestatic: Primary Biliary Cholangitis, Primary and Secondary Sclerosing Cholangitis. NASH: Non-alcoholic steatohepatitis; CSPH: Clinically significant portal hypertension defined as presence of at least one of the following: Ascites, gastroaesophaguel varices or hepatic encephalopathy.

(CI: 0.21-0.40) with a median survival of 15 mo (IQR 7-25 mo), when compared with BCLC-B without TACE and BCLC-C or D patients treated with TACE (Figure 2A). According to tumor reassessment after the first TACE, patients with complete response (CR) achieved a better overall survival with a HR of 0.15 (CI: 0.04-0.56. P = 0.005) (Figure 2B).

Characteristics and management of patients treated with sorafenib

Table 3 describes baseline patient characteristic treated with sorafenib (n = 82). Of these, 43.9% were BCLC-B, 43.9% BCLC-C and 12.2% BCLC-D. Among BCLC-B, 15 were TACE naïve and 21 received a median number of 3 TACE sessions (IQR 2-4 sessions) until disease progression (n = 7) or no response (n = 14). Among BCLC-C patients (n = 65), 55.4% were treated with sorafenib, 21 received BSC and 8 patients received other treatments (4 patients TACE, 1 TARE and patients 3 LR).

Median sorafenib treatment duration was 4.0 mo (IQR 2-11 mo). The most frequent sorafenib starting dose was 400 mg/d in 41% of the patients, followed by 800 mg/d in 32%. During follow-up, 55% of the patients achieved 800 mg full-dose of treatment, 35.4% had dose reductions (n = 29) of which in 21 patients dose-reduction were associated with drug-related adverse events. Most frequent adverse events (AEs) were fatigue (n = 27), diarrhea (n = 16), dermatologic events (n = 5), hand-foot-skin reaction (n = 3), and hypertension (n = 1). Permanent treatment discontinuation was observed in 12.2% of the patients secondary to treatment AEs (n = 10), tumor progression in 26.8%, (n = 22) and death in the rest of the patients. In 37 out of 82 patients in which radiologic evaluation after sorafenib initiation was performed, complete and partial responses were observed in 1.2% (n = 1) and 2.4% (n = 2), respectively. In these subgroup, median time to progression since sorafenib initiation was 7.3 mo (IQR 2.1-10.7 mo).

Corresponding median survival in all patients treated with sorafenib was 4.5 mo (IQR 2.3-11.7 mo); 5.2 mo (IQR 3.7-12.6 mo) in BCLC-B, 3.8 mo (IQR 1.9-9.9 mo) in BCLC-C and 3.2 mo (IQR 2.0-14.1 mo) in BCLC-D (Figure 3). When comparing BCLC-

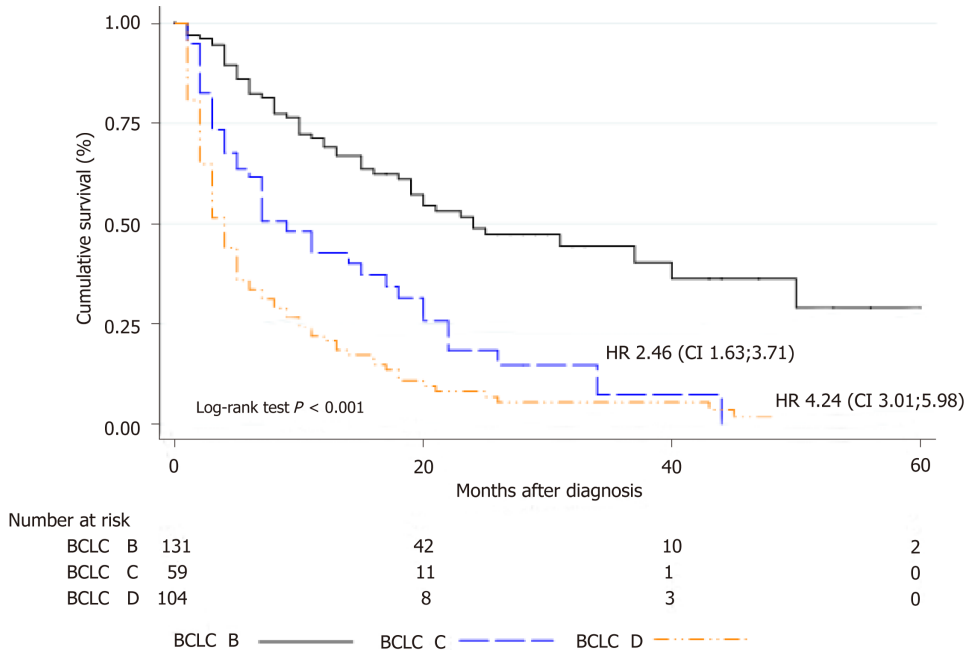

Figure 1 Cumulative survival stratified by Barcelona Clinic Liver Cancer staging in the overall cohort. BCLC: Barcelona clinic liver cancer.

B and C *vs* BCLC-D treated patients, although it did not reach statistical significance, a better survival curve was observed in BCLC-B/C patients with a HR of 0.63 (CI: 0.31-1.27; $P = 0.19$).

Sequential treatment with Sorafenib after TACE and impact on survival

Imaging evaluation after the first TACE in BCLC-B patients was registered in 64 out of 77 patients in median time from TACE to evaluation of 5 wk (IQR 4-6 wk). According to RECIST 1.1 criteria tumor response was as follows: partial response in 62.5% ($n = 40$), stable disease 15.6% ($n = 10$), complete response in 12.5% ($n = 8$), and disease progression in 9.3% ($n = 6$). Thus, overall objective response (ORR) and disease control rates (DCR) were 75% and 90.7% after first TACE, respectively.

In BCLC-B patients treated with sorafenib after progression ($n = 36$), the sequential treatment of sorafenib following TACE presented better survival when compared to those patients who received sorafenib without prior treatment with TACE [HR = 0.26 (CI: 0.09-0.71); $P = 0.013$] (Figure 4). Median number of TACEs in these patients prior to systemic treatment was 3 sessions (IQR 2-4 sessions). Among those patients not treated with TACE prior to sorafenib initiation, prior treatments were RFA/PEI ($n = 4$) and LR ($n = 5$).

DISCUSSION

This is the first observational study of treatments performed in the real life setting from Argentina in patients with intermediate to advanced stage HCC and one of the only ones to report post-treatment survival in Latin America. Knowing the real life treatment patterns is of interest to highlight unmet needs in the daily practice prior to the introduction of new treatments for HCC.

In this cohort we observed that in the majority of patients in intermediate stage, the most frequent treatment in daily practice was TACE. The effect on survival was beneficial in these patients in particular when treatment was established in accordance with Western clinical practice recommendations[6,7]. In patients with unpreserved liver function or BCLC-C, TACE was performed in a smaller proportion with heterogeneous effect on survival. On the other hand, those patients in BCLC-B stage with complete tumor response after TACE showed a better survival. Likewise, a non-negligible proportion of BCLC-B patients started sorafenib in the absence of prior TACE as a decision of "*treatment stage migration*"[10]. In the era of sequential treatment recommendation, in those BCLC-B patients with tumor progression after TACE[11], a better survival with sorafenib was observed with respect to those patients without prior TACE.

Knowing the therapeutic decisions in the daily practice is important because it reflects the gaps between interventional studies evaluating efficacy in *ideal situations*

Table 2 Stratified analysis comparing transarterial chemoembolization treatment in barcelona clinic liver cancer stage B

Variable	BCLC stage B overall (*n* = 135)	BCLC stage B with TACE (*n* = 77)	BCLC stage B without TACE (*n* = 58)	*P* value
Age, yr (± SD)	65 ± 10	65 ± 8	65 ± 11	0.86
Male gender, *n* (%)	111 (82.2)	68 (88.3)	43 (74.1)	0.03
Non-cirrhotic liver, *n* (%)	23 (17.0)	7 (9.1)	16 (27.6)	0.006
Etiology, *n* (%)				0.11
HCV	42 (31.1)	25 (32.5)	17 (29.3)	
HBV	8 (5.9)	5 (6.5)	3 (5.2)	
Alcohol	29 (21.5)	21 (27.3)	8 (13.8)	
Etiology, *n*NASH	14 (10.4)	6 (7.8)	8 (13.8)	
Etiology, *n*Others	42 (31.1)	20 (25.9)	22 (37.9)	
Child Pugh A/B, *n* (%)	88 (65.2)/47 (34.8)	48 (62.3)/29 (37.7)	40 (69.0)/18 (31.0)	0.42
CSPH[1], *n* (%)	67 (49.6)	45 (58.4)	22 (37.9)	0.018
Median *n*° HCC nodules (IQR)	2 (2-3)	2 (1-3)	2 (1-4)	0.39
Median largest HCC diameter, mm, (IQR)	65 (43-100)	60 (43-88)	69.5 (45-114.5)	0.11
Bilobar involvement, n (%)	53 (39.3)	30 (39.0)	23 (39.7)	0.72
Diffuse HCC pattern, n (%)	5 (3.7)	2 (2.6)	3 (5.2)	0.72
Median AFP, ng/mL (IQR)	26.7 (4.7-248.5)	27.5 (5.1-202.85)	24.4 (4.3-285)	0.91
AFP > 200 ng/mL, *n* (%)	36 (27.3)	19 (25.0)	17 (30.4)	0.49
AFP > 400 ng/mL, *n* (%)	30 (22.7)	17 (22.4)	13 (23.2)	0.91
AFP > 1000 ng/mL, *n* (%)	18 (13.6)	11 (14.5)	7 (12.5)	0.74
Vascular invasion, *n* (%)	-			
Extrahepatic disease, *n* (%)	-			

[1]Clinically significant portal hypertension defined as presence of at least one of the following: Ascites, gastro-aesophageal varices or hepatic encephalopathy. AFP: Alpha-feto protein; TACE: Transarterial chemoembolization; BCLC: Barcelona clinic liver cancer.

and those in the *real-life* setting. The BRIDGE study is an example, among others, of how therapeutic decisions in patients with HCC are complex, demanding a fine knowledge not only of tumor extension, but also of liver disease and its complications[12]. That is why the role of hepatologists is of utmost importance in the treatment of these patients. In our cohort, most of the patients were screened, diagnosed and treated by hepatologists, both in referral or local centers.

Treatment with TACE has been established as the gold standard for intermediate stage HCC since more than 10 years ago[3,4]. Two randomized, placebo-controlled trials have shown its survival benefit[3,4]; results further underlined in a meta-analysis[13]. However, clinical and tumor heterogeneity, which are characteristic of BCLC-B patients, results in a diversity of established treatments[12]. In our analysis, we excluded BCLC-B patients who underwent transplantation, given that we considered performing a *pure* analysis in this stage. The same went for BCLC-D patients. In the original trials of TACE, a median survival was close to eighteen months[3,4,14] whereas in more recent observational studies, median survival of forty months has been reported[15]. In our study, median survival in BCLC-B patients treated with TACE was fifteen months. Survival was significantly better in BCLC-B patients treated with TACE with a 71% relative risk reduction of death when compared with BCLC-B without TACE and BCLC-C or D patients treated with TACE. According to tumor reassessment by RECIST 1.1 criteria after the first TACE, patients with complete response had the highest survival benefit, as previously reported elsewhere[16].

Systemic treatment of HCC is remarkably changing given the introduction of alternative therapies in first line such as lenvatinib[17] and second-line including regorafenib[18], cabozantinib[19] and ramucirumab[20]. In our country, as in many others from Latin America, approval of these new treatments usually takes between 12 and 24 mo later than other developed regions of the world. In 2009 and 2017, sorafenib and regorafenib were approved in our country, respectively. Recently, the use of lenvatinib has also been approved, not yet included in the daily practice. Treatment with immunotherapy, either with nivolumab[21] or pembrolizumab[22], has not been approved by the National Regulatory Agency in our country (ANMAT).

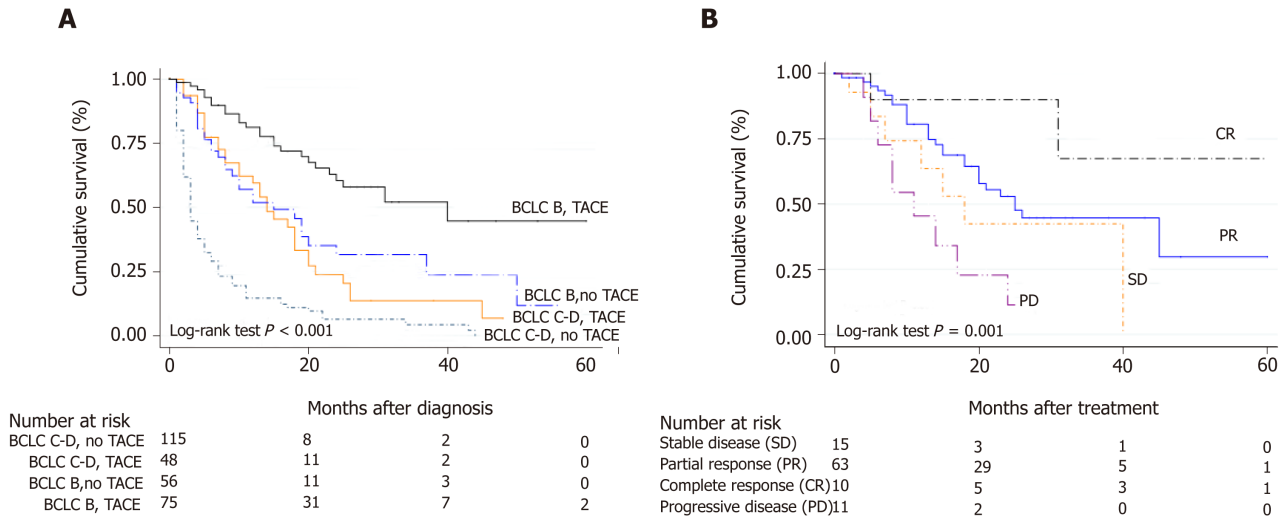

Figure 2 Characteristics and management of patients treated with transarterial chemoembolization. A: Kaplan Meier survival curves according to Barcelona Clinic Liver Cancer stage and treatment with/without trans-arterial chemoembolization; B: Survival according to radiological response after the first transarterial chemoembolization (TACE) evaluated by RECIST 1.1 criteria. BCLC: Barcelona clinic liver cancer.

Argentina is a South American country with a wide extension, a great socio-cultural heterogeneity with a large variety in health care systems. In many cases, the main barrier for the access to health system is the authorization by insurances to carry out diagnostic studies or therapies due to costs or other barriers. This problem is common in Latin America[23]. In this study, the use of sorafenib slightly exceeded half of BCLC-C patients, presenting better survival when compared with those patients in the same stage but without systemic treatment. We observed that in our cohort, median survival with the use of sorafenib was strikingly low, being no more than 5 months. This lower than expected outcome can be explained, in part, by the delay in starting treatment, due to a wide range of authorizations and complex administrative processes. This might have leaded to a significant slowness in the initiation of systemic treatment. Moreover, most of the patients were initially treated with half dose rather than full dose.

It is noteworthy of mention that sorafenib tolerance was similar to that reported from first (SHARP and Asia-Pacific)[5,24] and second line (RESORCE)[18] clinical trials, with a rate of definitive treatment discontinuation due to related adverse events of 12.2%. On the other hand, in those patients in whom radiological response was evaluated, median time to progression under treatment with sorafenib was similar than that previously reported[18]. Finally, we observed that there was an inadequate use of sorafenib in patients with unpreserved liver function or BCLC-D that was associated with a poor prognosis, demonstrating an inadequate and inefficiency use of resources.

Our study has limitations. In particular, given that it was mainly a retrospective cohort study, exposed to different selection and information biases. Specifically, neither radiological evaluation assessing time to progression was homogeneous nor there was a centrally blinded evaluation through all participating centers. However, we enrolled a group of centers presenting similar decision making processes trying to homogenize the sample.

In conclusion, in this dual cohort study from Argentina, we described the treatments performed in the real life setting before the approval of new systemic options. Knowing this life context is of interest, in order to assess the most common therapeutic decision making processes and management in these patients. In this real setting, our results highlights unmet needs and improvement areas in public health among developing regions, particularly to promote early and correct treatments in each stage, prior to the introduction of new treatments for HCC.

ACKNOWLEDGEMENTS

On behalf of the Latin American Liver Research, Education and Awareness Network (LALREAN).

Table 3 Characteristic of patients treated with sorafenib

Variable	Overall (*n* = 82)	BCLC stage B (*n* = 36)	BCLC stage C (*n* = 36)	BCLC stage D (*n* = 10)	*P* value
Age, yr (± SD)	63 ± 9	63 ± 8	62 ± 10	63 ± 8	0.86
Male gender, *n* (%)	68 (82.9)	29 (80.6)	31 (86.1)	8 (80.0)	0.88
Non-cirrhotic liver, *n* (%)	9 (11.0)	3 (8.3)	5 (13.9)	1 (10.0)	0.78
Etiology, *n* (%)					0.11
HCV	28 (34.1)	16 (44.4)	11 (30.6)	1 (10.0)	
HBV	4 (4.9)	2 (5.6)	1 (2.8)	1 (10.0)	
Alcohol	18 (21.9)	9 (25.0)	8 (22.2)	1 (10.0)	
NASH	10 (12.2)	4 (11.1)	4 (11.1)	2 (20.0)	
Others	22 (26.8)	5 (13.9)	12 (33.3)	5 (50.0)	
Child Pugh A/B/C, *n* (%)	48 (58)/30 (37)/4 (5)	25 (69)/11 (31)/-	21 (58)/15 (42)/-	2 (20)/4 (40)/4 (40)	
CSPH[1], *n* (%)	45 (54.9)	21 (58.3)	18 (50.0)	6 (60.0)	0.66
Median nº HCC nodules (IQR)[2]	2 (1-4)	2 (2-4)	2 (1-4)	1.5 (1-2)	0.25
Median largest HCC diameter, mm, (IQR)[2]	70 (47-100)	65 (46-90)	87 (48.5-130)	57.5 (39-121)	0.56
Bilobar involvement, *n* (%)	30 (37.0)	10 (27.8)	18 (50.0)	3 (30.0)	0.33
Diffuse HCC pattern, n (%)	6 (7.4)	2 (5.6)	3 (8.3)	1 (10.0)	0.35
Median AFP, ng/mL (IQR)	103 (7.0-1069)	30 (7.2-739)	150 (6.3-1210)	649 (16-2198)	0.26
AFP > 200 ng/mL, *n* (%)	35 (43.7)	12 (34.3)	16 (45.7)	7 (70)	0.13
AFP > 400 ng/mL, *n* (%)	30 (37.5)	10 (28.6)	13 (37.1)	7 (70)	0.06
AFP > 1000 ng/mL, *n* (%)	21 (25.9)	5 (14.3)	12 (33.3)	4 (40)	0.13
Vascular invasion, *n* (%)	27 (33.3)	-	25 (69.4)	3 (30.0)	< 0.0001
Extrahepatic disease, *n* (%)	19 (23.5)	-	18 (50.0)	1 (10.0)	< 0.0001

[1]Clinically significant portal hypertension defined as presence of at least one of the following: ascites, gastro-aesophageal varices or hepatic encephalopathy.
[2]Intrahepatic nodules. AFP: Alpha-feto protein; TACE: Transarterial chemoembolization; BCLC: Barcelona clinic liver cancer.

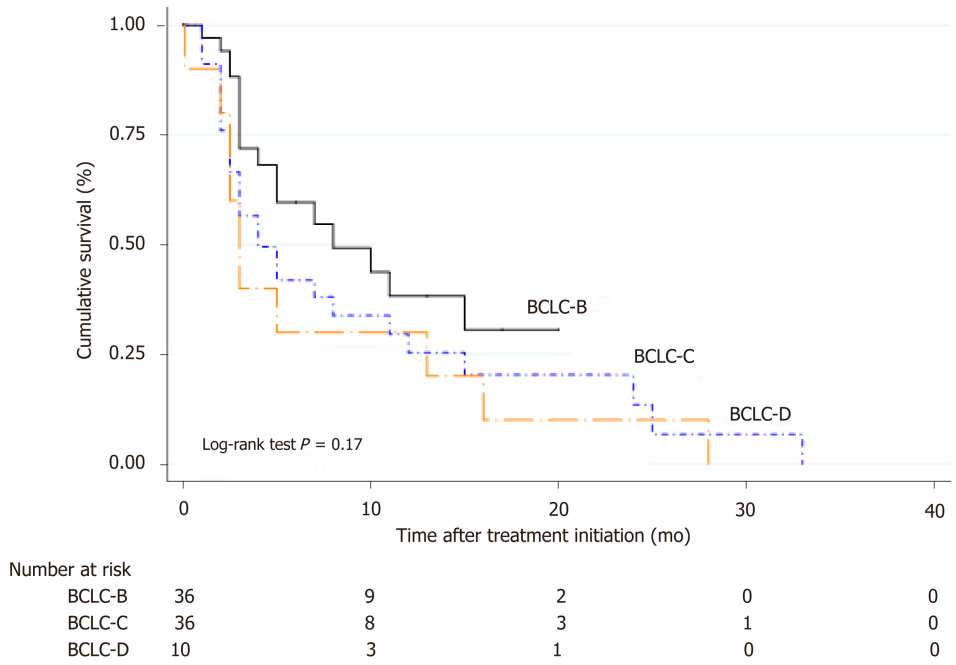

Figure 3 Corresponding survival curves for patients treated with Sorafenib stratified by Barcelona Clinic Liver Cancer stages. BCLC: Barcelona clinic liver cancer.

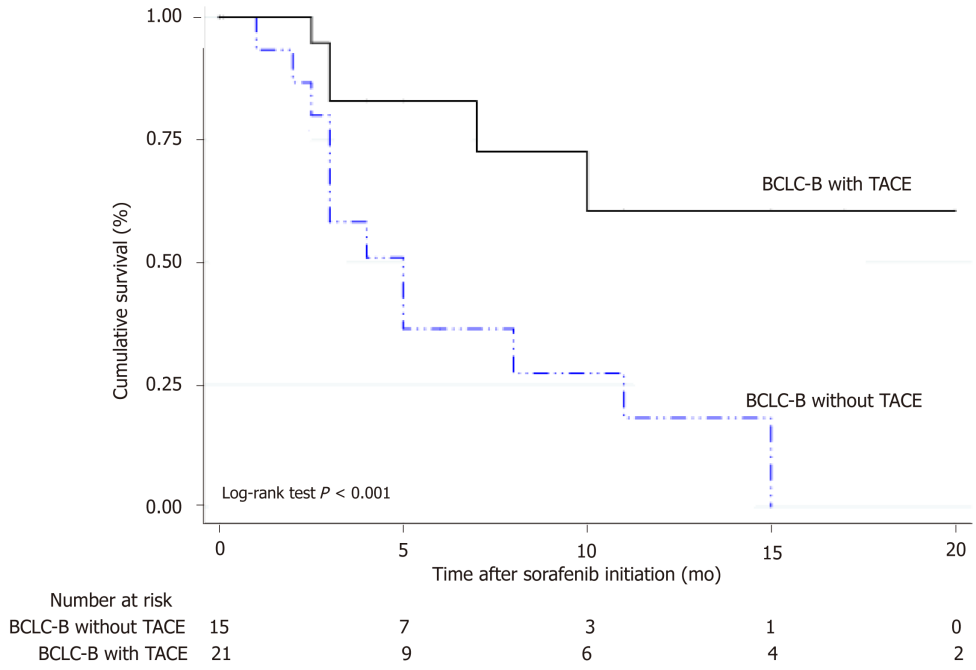

Figure 4 Survival in Barcelona Clinic Liver Cancer stage B patients under tumor progression with the sequential treatment of transarterial chemoembolization-sorafenib. BCLC: Barcelona clinic liver cancer.

ARTICLE HIGHLIGHTS

Research background

Although liver cancer or hepatocellular carcinoma (HCC) is currently the 5th most common cancer and the 2nd cause of death from cancer worldwide, in Argentina represents the sixteenth most frequent cancer. Transarterial chemoembolization (TACE) and systemic treatment with sorafenib are the standards of treatment for patients with intermediate and advanced stage HCC.

Research motivation

The rise of new therapeutic modalities such as radioembolization, the combination of antiangiogenic agents with locoregional therapies and other first and second line systemic options, open up a new paradigm for the treatment of HCC.

Research objectives

Our aim was to describe the treatments performed in the real life setting before the approval of these new systemic options.

Research methods

This longitudinal observational cohort study was conducted between in 14 different regional hospitals from Argentina between 2009 and 2016. Study data were registered into a web-based electronic system. Patients with intermediate (BCLC-B) or advanced (BCLC C-D) HCC were included. Patients were excluded if (1) clinical baseline data was missing; (2) BCLC stage was either 0 or A, in which potentially curative treatments are recommended such as liver resection (LR), percutaneous ethanol injection (PEI)/radiofrequency ablation (RFA) or liver transplantation (LT); and (3) patients with BCLC-B-D who underwent liver transplantation. Baseline tumor and patients characteristics at HCC diagnosis, as well as treatments performed were registered. Each treatment was discussed at each center on a case-by-case basis. Imaging tumor reassessment after treatments were done according to RECIST 1.1 criteria as recommended by international Western guidelines. Median survival was assessed for each BCLC stage from the date of treatment until last patient follow-up or death. For survival analysis, Cox regression analysis estimating hazard ratios (HR) and 95%CI for baseline variables related with mortality was performed. Kaplan Meier survival curves were compared using the log-rank test (Mantel-Cox).

Research results

A total of 327 consecutive adult patients with intermediate and advanced HCC were included, of which 41.3% of the patients were in BCLC stage B (n = 135), 19.9% in stage C (n = 65) and 38.8% in stage D (n = 127). Corresponding median survival for BCLC stages were as follows: Stage B 15 mo (IQR 5-26 mo), stage C 5 mo (IQR 2-13 mo) and stage D 3 mo (IQR 1-13 mo)(Figure 1). TACE was performed in 126 patients (38.5%); 77 were BCLC-B, 22 were BCLC-C and 27 patients were BCLC-C. Among BCLC-B patients (n = 135), 57% received TACE (n = 77) whereas 43% did not (Table 2). Median number of TACEs sessions was 2 (IQR 1-3 sessions). Survival was significantly

better in BCLC-B patients treated with TACE HR 0.29 (CI: 0.21-0.40) with a median survival of 15 mo (IQR 7-25 mo), when compared with BCLC-B without TACE and BCLC-C or D patients treated with TACE. According to tumor reassessment after the first TACE by RECIST 1.1 criteria, patients with complete response (CR) achieved a better overall survival with a HR of 0.15 (CI: 0.04-0.56, P = 0.005). Table 3 describes baseline patient characteristic treated with sorafenib (n = 82). Of these, 43.9% were BCLC-B, 43.9% BCLC-C and 12.2% BCLC-D. Among BCLC-B patients who received sorafenib, 15 were TACE naïve and 21 received a median number of TACEs of 3 (IQR 2-4) until disease progression (n = 7) or no response or un-TACE-able (n = 14). Among BCLC-C patients (n = 65), 55.4% were treated with sorafenib and those not treated with sorafenib received BSC (n = 21) and other treatments (4 patients TACE, 1 TARE and patients 3 LR). Corresponding median survival in all patients treated with sorafenib was 4.5 mo (IQR 2.3-11.7 mo); 5.2 mo (IQR 3.7-12.6 mo) in BCLC-B, 3.8 mo (IQR 1.9-9.9 mo) in BCLC-C and 3.2 mo (IQR 2.0-14.1 mo) in BCLC-D. In BCLC-B patients treated with sorafenib after progression (n = 36), the sequential treatment of sorafenib following TACE presented better survival since systemic treatment when compared to those patients who received sorafenib without prior treatment with TACE [HR = 0.26 (CI: 0.09-0.71); P = 0.013].

Research conclusions

In conclusion, in this dual cohort study from Argentina, we describe the treatments performed in the real life setting before the approval of new systemic options.

Research perspectives

Knowing the real life setting is of interest, in order to assess the most common therapeutic decision making processes and management in these patients. Our results highlights unmet needs and improvement areas in public health among developing regions such as Argentina, particularly to promote early and correct treatments in each stage, prior to the introduction of new treatments for HCC.

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ORIGINAL ARTICLE

Retrospective Study

Quantitative diffusion-weighted magnetic resonance enterography in ileal Crohn's disease: A systematic analysis of intra and interobserver reproducibility

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Abstract

BACKGROUND

Magnetic resonance enterography (MRE) is increasingly attractive as a noninvasive and radiation-free tool for assessing Crohn's disease (CD). Diffusion-weighted imaging (DWI) is recommended as an optional MRE sequence for CD by the European Society of Gastrointestinal and Abdominal Radiology, and has shown a superb potential as a quantitative modality for bowel inflammation evaluation. However, the measurement reproducibility of quantitative DWI analysis in MRE has not been ascertained so far. To facilitate the application of quantitative diffusion-weighted MRE in the clinical routine, systematic investigations of the intra and interobserver reproducibility of DWI quantitative parameters should be performed.

AIM

To evaluate the intra and interobserver reproducibility of quantitative analysis for diffusion-weighted MRE (DW-MRE) in ileal CD.

METHODS

Forty-four subjects (21 with CD and 23 control subjects) who underwent ileocolonoscopy and DW-MRE (b = 800 s/mm^2) within one week were included. Two radiologists independently measured apparent diffusion coefficients (ADC) of the terminal ileum and signal intensity ratio (SR) of the terminal ileum to ipsilateral psoas muscle on DWI images (b = 800 s/mm^2). Between- and within-reader agreements were assessed using intraclass correlation coefficients (ICC), coefficients of variation (CoV), and 95% limits of agreement of Bland-Altman plots (BA-LA LoA). Diagnostic performances of ADC and SR for identifying

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inflamed terminal ileum from the normal were evaluated by receiver operating characteristic (ROC) curve analysis.

RESULTS

There were no significant differences in ADC or SR values between the two sessions or between the two radiologists either in the CD or control group (paired *t*-test, $P > 0.05$). The intra and interobserver reproducibility of ADC (ICC: 0.952-0.984; CoV: 3.73-6.28%; BA-LA LoA: ±11.27% to ±15.88%) and SR (ICC: 0.969-0.989; CoV: 3.51%-4.64%; BA-LA LoA: ±10.62% to ±15.45%) was excellent for CD. Agreement of ADC measurements was slightly less in control subjects (ICC: 0.641-0.736; CoV: 10.47%-11.43%; BA-LA LoA: ± 26.59% to ± 30.83%). SR of normal terminal ileum demonstrated high intra and interobserver reproducibility (ICC: 0.944-0.974; CoV: 3.73%-6.28%; BA-LA LoA: ± 18.58% to ± 24.43%). ADC and SR of two readers had outstanding diagnostic efficiencies (area under the ROC curve: 0.923-0.988).

CONCLUSION

Quantitative parameters derived from DW-MRE have good to excellent intra and interobserver agreements with high diagnostic accuracy, and can serve as robust and efficient quantitative biomarkers for CD evaluation.

Key words: Magnetic resonance imaging; Diffusion-weighted imaging; Crohn's disease; Ileum; Reproducibility

Core tip: We evaluated the measured reproducibility of quantitative apparent diffusion coefficients (ADC) and signal contrast ratio (SR) of the terminal ileum to ipsilateral psoas muscle on diffusion-weighted imaging (DWI; b = 800 s/mm²). The intra and interobserver reproducibility of ADC and SR values was good to excellent for Crohn's disease (CD) and normal control subjects. Quantitative parameters of ADC and SR derived from DWI had outstanding diagnostic efficacy for CD. Therefore, both ADC and SR could serve as robust and efficient quantitative biomarkers for the evaluation of CD.

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INTRODUCTION

Crohn's disease (CD) is a life-long, relapsing, chronic inflammatory disease of the gastrointestinal tract that most frequently involves the terminal ileum[1]. Serial evaluation including monitoring of disease and assessment of treatment efficacy is important throughout the patient's life[2-4]. For non-invasive assessment, magnetic resonance (MR) enterography (MRE) is increasingly attractive as an ionizing radiation-free supplement to endoscopy in assessment of CD patients[5-8]. MRE presents several advantages as it not only assesses abnormalities of the bowel lumen[5], but also identifies extra-intestinal complications (abscesses or fistulae)[2,6], offers an assessment of the whole gastrointestinal tract[8], and has the merit owing to its high soft tissue contrast[9,10]. Since the conventional MRE is based upon the qualitative morphological assessment of a lesion, an accurate quantitative MR technique may enable a more objective assessment of clinical therapeutic effect[6,9].

As a promising functional MR imaging (MRI) technique[7,9-12], diffusion-weighted imaging (DWI) could qualitatively and quantitatively evaluate random motion of water molecules at the microstructural level in biological tissues, and is recommended as an optional sequence for CD by the European Society of Gastrointestinal and Abdominal Radiology in the latest consensus statements[13]. Meanwhile, DWI has been shown to be an alternative to contrast-enhanced MRI for the detection of inflamed

segments in CD patients, and is suitable for the patients who cannot receive intravenous contrast agents[6,14]. Furthermore, the latest consensus recommendations pointed out that restricted diffusion on DWI was a valuable and supportive sign correlating with the inflammatory lesions demonstrated on endoscopy[15,16]. The apparent diffusion coefficient (ADC) derived from DWI has been reported as a potential quantitative biomarker for monitoring the course of disease and therapeutic efficiency in patients with CD[17].

However, ADC values calculated from DWI could be degraded by variations in field homogeneity, eddy currents, and motion, particularly in hollow organs like the gastrointestinal tract or gallbladder[5,18]. And systematic investigations on measurement reproducibility of quantitative DWI analysis in MRE have not been ascertained so far. The characterization of reader agreement and variability with quantitative parameters is imperative before such parameters are to be incorporated into clinical practice[19-21]. As ADC values could be affected by the image mismatch calculated from DWI with at least two b values[5,8], we proposed an abbreviated proxy for diffusion restriction, a signal intensity ratio (SR), between the terminal ileum and ipsilateral psoas muscle on DWI with an appropriate b value (800 s/mm^2), and explored the application of SR and ADC values in CD patients.

The purpose of this study was to investigate the level of inter and intraobserver agreement for ADC and SR measurements from DWI in the terminal ileum, and then to assess the diagnosis efficiency of quantitative ADC and SR for the evaluation of CD.

MATERIALS AND METHODS

Patients

The study was approved by our institutional review board, and informed consent was waived for the retrospective study design. From November 2015 to May 2018, a total of 297 patients underwent MRE examinations for suspected gastrointestinal disorders at our gastrointestinal medical center of the referral university-based hospital. Sixty-one patients were included based on the follow criteria: (1) Complete clinical data available; (2) DWI with a b-value of 800 s/mm^2 included in the MRE protocol; (3) Colonoscopy was successfully conducted to assess the terminal ileum within one week of the MR enterography; and (4) No bowel surgery performed prior to MRI examination. Among the 61 patients, 12 diagnosed with other diseases and 5 with inadequate MRE images due to serious artifacts were excluded. Finally, the remaining 44 patients, including 21 with CD involving the terminal ileum and 23 with a normal terminal ileum as control subjects, were included for the further analysis. A flowchart of the study population is shown in Figure 1. The diagnosis of CD was confirmed by clinical information and a combination of endoscopic, histological, and radiological investigations according to the European Crohn's and Colitis Organization criterion[22].

MRI data acquisition

MRE was performed in a supine position using a 3.0-T GE scanner (Discovery MR750; GE Healthcare, Milwaukee, WI, United States) with a 32-channel torso phased array body coil. Per protocol, all patients had fasted for at least 6 h before MRE. To achieve adequate distension of the small bowel, about 1500 mL of 2.5% isosmotic mannitol solution was administered orally to the patients 45 min prior to MR scanning. Anisodamine 10 mg was administered intramuscularly 5 min before the MR examination to reduce bowel peristalsis. Conventional MRE was acquired including T2-weighted single shot fast spin echo (SSFSE; TR/TE: a respiratory cycle/68 ms, matrix: 288 × 288, slice thickness: 4 mm for coronal/5 mm for axial), the fast imaging employing steady state acquisition (FIESTA; TR/TE: 3.2/1.2 ms, matrix: 288 × 288, slice thickness: 4 mm for coronal/5 mm for axial, flip angle: 45 degrees), and coronal and axial T1 weighted three-dimensional fast spoiled gradient echo (liver acquisition with volume acceleration sequence, LAVA; TR/TE: 3.8/1.7 ms, matrix: 260 × 210, slice thickness: 4 mm, flip angle: 15 degrees).

The axial DWI sequence was obtained using single shot echo planar imaging (SS-EPI; TR/TE: a respiratory cycle/minimum, field of view: 38.0 cm × 30.4 cm, matrix: 160 × 128, slice thickness: 6 mm) with b values of 0 and 800 s/mm^2; the number of excitations (NEX) was 6. The total acquisition time of the whole examination for every patient was approximately 25 min depending on the patient's respiratory rate.

Image analysis

All images were transferred to a workstation (Advantage Workstation 4.6, GE Healthcare) where ADC maps were generated by using a mono-exponential fit. Two

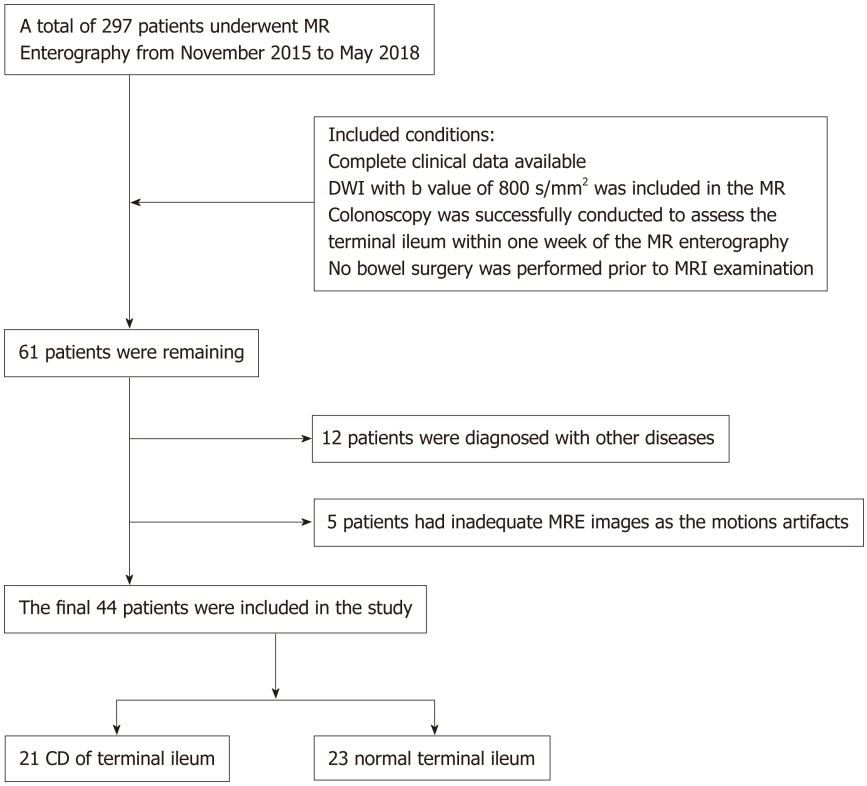

Figure 1 Flow chart illustrating study design. DWI: Diffusion weighted imaging; CD: Crohn's disease.

gastrointestinal radiologists (R1 with 5 years of experience and R2 with 3 years of experience), both blinded to clinical details as well as endoscopy results, measured the signal intensity (SI) of the terminal ileum (defined as the final 10 cm of the small bowel) and ipsilateral psoas muscle on DWI images with b = 800 s/mm^2. Separately and simultaneously, ADC values for the terminal ileum were recorded. For the SI and ADC measurements in the terminal ileum, the images were magnified, and three oval regions of interest (ROI), as large as possible (12-30 mm^2) to encompass the bowel wall, were placed on the area of brightest signal on the DWI along with hypointensity on ADC maps (Figure 2), as having been described in previous studies[9,23]. The means of the three measurements (SI and ADC) were used for the further analysis. Then, SI of the psoas muscle on DWI with b = 800 s/mm^2 was measured by placing a larger oval ROI (60-100 mm^2). The SR of the terminal ileum to ipsilateral psoas muscle was calculated according to the prior studies[24,25] by the equation: $SR = SI_{ileum}/SI_{muscle}$, where SI_{ileum} and SI_{muscle} represent the measured SI of the terminal ileum and ipsilateral psoas muscle, respectively. After a washout period of 4 wk, the same two radiologists repeated the above described measurements to assess intraobserver agreement.

Reference standard: Endoscopy assessment

The terminal ileum of all the subjects was assessed by ileocolonoscopy as the reference standard. Prior endoscopy, bowel preparation was performed *via* oral ingestion of about 4 L of polyethylene glycol solution according to the standard protocol. Endoscopy was successfully conducted to assess the terminal ileum in all of the included patients. Endoscopic analysis of the terminal ileum was performed by an endoscopist with 10 years of experience who was blinded to MRE results and clinical data. The colonoscopy findings of the terminal ileum, such as overt ulcers, aphthoid lesions, and erythema or edema, were considered as inflammatory conditions[17,22].

Statistical analysis

Statistical calculations were performed using the Statistical Package for the Social Sciences Software Package (SPSS Statistics, version 20.0; SPSS Inc., Chicago, IL, United States) and MedCalc (release 15.2.2; MedCalc Software, Mariakerke, Belgium). The normal distribution of quantitative parameters was ascertained by the Kolmogorov-Smirnov test. Differences in ADC and SR between the two readers and two sessions within the CD and control groups were compared using a paired *t* *t*-test. Inter and intraobserver agreements for SR and ADC measurements were assessed using the intraclass correlation coefficient (ICC), coefficients of variation (CoV) from duplicate

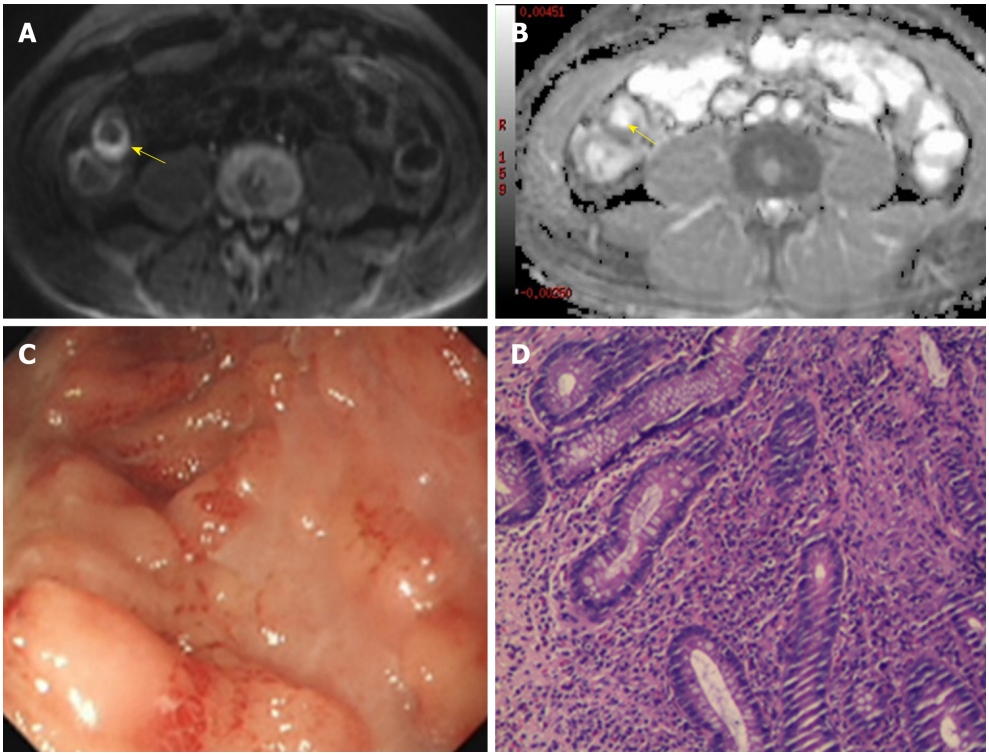

Figure 2 A 48-year-old man with Crohn's disease involving the terminal ileum. A: Axial diffusion-weighted imaging with b = 800 s/mm^2 demonstrated high signal intensity of the terminal ileum (arrow). B: The terminal ileum was hypointense (arrow) on the corresponding apparent diffusion coefficient map. C: Ileocolonoscopy showed mucosal ulcers. D: Histopathology demonstrated neutrophil infiltration.

measurements, and the Bland-Altman analysis with 95% limits of agreement (LoA). ICC was interpreted as follows: 0.00-0.20, poor; 0.21-0.40, fair; 0.41-0.60, moderate; 0.61-0.80, good; 0.81-1.00, excellent. Diagnostic efficiency of the DWI parameters was determined by receiver operating characteristic (ROC) curve analyses. The comparison of the areas under ROC curve (AUROC) was performed between SR and ADC using the method of Delong *et al*. Spearman's correlation test was used to identify correlation between SR and ADC values in CD patients. *P*-values less than 0.05 were considered statistically significant.

RESULTS

Patient characteristics

A total of 44 subjects (age: Mean ± SD, 44.9 ± 15.7 years; 22 males and 22 females) were included in this study, and all of the DWI images were eligible for evaluation. Twenty-three control subjects had a normal terminal ileum in colonoscopy examination. The remaining 21 CD patients with the terminal ileum involved presented 12 overt ulcers, 5 aphthoid lesions, and 4 erythema or edema conditions according to endoscopy. The patients' data of this cohort are summarized in Table 1.

Comparisons of quantitative ADC and SR between two reading sessions or two radiologists

There were no significant differences (paired *t*-test, $P > 0.05$) in ADC or SR values between the two sessions or between the two radiologists either in the CD or control group (Tables 2 and 3). The intra and interobserver agreements of quantitative DWI parameters measurements are shown in Tables 2 and 3 and Figures 3 and 4.

Intraobserver agreement analysis

The intraobserver agreement for SR was excellent in both the CD and control groups with similar results obtained by the two readers (R1: ICC = 0.989, CoV = 3.51%; R2: ICC = 0.979, CoV = 4.64% for CD and R1: ICC = 0.974, CoV = 5.93%; R2: ICC = 0.944, CoV = 8.69% for the normal). Similarly, the ADC measurements in the CD group showed excellent intraobserver reproducibility (R1: ICC = 0.952, CoV = 6.28%; R2: ICC = 0.977, CoV = 4.71%). However, intraobserver agreement was inferior when measuring the ADC on the normal terminal ileum (R1: ICC = 0.736, CoV = 10.47%; R2:

Table 1 Clinical characteristics of included subjects

Characteristic	Value
CD patients (*n*)	21
Age (yr) (mean ± SD)	43.4 ± 16.1
Gender (males: Females)	11:10
CRP (mg/dL) [median (range)]	21.0 (0.9-94.0)
ESR (mm/h) [median (range)]	19 (7-51)
Days between MRE and endoscopy median [median (range)]	4 (0-7)
Endoscopic findings of terminal ileum (*n*)	
Overt ulcers	12
Aphthoid lesions	5
Erythema or edema conditions	4
Control group (*n*)	23
Age (yr) (mean ± SD)	46.3 ± 15.4
Gender (males:females)	11:12
Days between MRE and endoscopy [median (range)]	3 (1-7)
Total (*n*)	44
Age (yr) (mean ± SD)	44.9 ± 15.7
Gender (males:females)	22:22
Days between MRE and endoscopy [median (range)]	4 (0-7)

CD: Crohn's disease; SD: Standard deviation; CRP: C-reactive protein; ESR: Erythrocyte sedimentation rate; MRE: Magnetic resonance enterography.

ICC = 0.711, CoV = 10.64%). Bland-Altman plots for intraobserver agreement showed rather narrow 95% LoA ranges except for the ADC measurements in the control group; no systematic bias between sessions was present (Figure 3).

Interobserver agreement analysis

Similar to the analysis of intraobserver reproducibility, all ADC measurements in the CD group and SR in both the CD and normal groups demonstrated excellent interobserver agreement (Table 3). The lowest agreement and highest variability between readers were observed in ADC measurements of the control group (first session: ICC = 0.641, CoV = 11.43%; second session: ICC = 0.736, CoV = 11.07%). Similarly, only the interreader ADC measurements for the control group demonstrated broad 95% LoA ranges (-33.93% to 25.96% for first session; -32.03% to 29.62% for second session) as illustrated in Figure 4.

Spearman's correlation analysis between SR and ADC values in the CD patients

The correlation between the quantitative DWI parameters of SR and ADC is shown in Figure 5. SR was moderately and negatively correlated with ADC values in CD patients for both readers (R1: r = - 0.438, P = 0.047; R2: r = - 0.485, P = 0.026).

Diagnostic efficiency of ADC and SR for differentiating inflammatory bowel segments from the normal

Due to the good to excellent intraobserver agreement of ADC and SR measurements in two sessions of the two readers, the mean ADC and SR values were calculated based on an average from the two sessions for further analysis. ADC and SR from both readers achieved a high diagnostic accuracy for differentiating inflammatory segments from the normal bowel with high sensitivities and specificities (Table 4 and Figure 6). The areas under the ROC curve for SR (R1: 0.981; R2: 0.988) were slightly better than those of ADC (R1: 0.923; R2: 0.929), but these differences were not statistically significant (P-values: 0.117 for R1; 0.067 for R2). The cutoff values maximizing sensitivity and specificity for ADC and SR parameters were approximately 1.80 × 10^{-3} mm^2/s and 2.00, respectively.

DISCUSSION

DW-MRE has been increasingly utilized for the noninvasive evaluation of CD over the last few years[5-10,14,26]. Meanwhile, quantitative analysis of DW-MRE is gradually

Table 2 Intraobserver agreement and variability of apparent diffusion coefficient and signal ratio measurements between the two readers over two sessions

Group	CD (*n* = 21)						Normal (*n* = 23)					
	1st session reader 1	2nd session reader 1	*P*-value	1st session reader 2	2nd session reader 2	*P*-value	1st session reader 1	2nd session reader 1	*P*-value	1st session reader 2	2nd session reader 2	*P*-value
ADC	1.50 ± 0.40	1.54 ± 0.45	0.201	1.52 ± 0.45	1.56 ± 0.47	0.125	2.55 ± 0.53	2.53 ± 0.53	0.772	2.57 ± 0.50	2.64 ± 0.57	0.450
ICC	0.952			0.977			0.736			0.711		
CoV	6.28			4.71			10.47			10.64		
Bias (%)	1.77			1.89			-1.07			-1.71		
LoA (%)	-14.10 to 17.65			-10.65 to 14.42			-30.75 to 28.62			-28.30 to 24.86		
SR	4.17 ± 1.43	4.20 ± 1.36	0.505	4.21 ± 1.34	4.23 ± 1.42	0.794	1.45 ± 0.54	1.44 ± 0.53	0.697	1.44 ± 0.52	1.45 ± 0.56	0.704
ICC	0.989			0.979			0.974			0.944		
CoV	3.51			4.64			5.93			8.69		
Bias (%)	1.39			-0.07			-0.58			0.45		
LoA (%)	-9.22 to 12.01			-13.81 to 13.67			-19.16 to 17.99			-23.99 to 24.88		

Apparent diffusion coefficient (ADC) and signal ratio parameters are expressed as the mean ± standard deviation, ADC units: $\times 10^{-3}$ mm^2/s. ADC: Apparent diffusion coefficient; SR: Signal ratio; CD: Crohn's disease; ICC: Intraclass correlation coefficient; CoV: Coefficients of variation; LoA: Bland-Altman analysis with 95% limits of agreement.

drawing attention as an objective biomarker for bowel inflammation[9,17,26-28]. ADC values along with the Clermont score have been reported as having excellent accuracy for identifying inflamed bowel segments and evaluating therapeutic response in CD patients[17,23,26]. However, the measurement agreement for DW-MRE quantitative parameters has not been ascertained to date[8]. To facilitate the application of quantitative DW-MRE into the clinical routine, a thorough study of the intra and interobserver reproducibility of DWI quantitative parameters should be performed, similar to what has been done in other organs[19,20].

In the current study, intra and interobserver agreements of ADC measurements in CD patients were excellent with high ICC, low CoV, and a limited 95% LoA range (Tables 2 and 3). ADC values of the terminal ileum in the CD group were robust both between and within individual readers, suggesting that different radiologists could obtain comparable ADC values in CD patients over time. In previous studies[9,23,29], interobserver agreement of measured ADC values in CD patients was also superb with high ICC, similar to our results of ADC analysis between radiologists. As the same with ADC values, excellent intra and interobserver agreements of SR measurement were found in inflamed terminal ileum. As expected, SR demonstrated moderately negative correlations with ADC in CD patients in both readers (Figure 5), indicating that SR could also represent the diffusion condition on DWI just like the ADC value. Therefore, both ADC and SR could act as the robust and quantitative methods for the evaluation of CD patients.

Although the comprehensive Clermont score has been introduced as a valuable tool for the ileal CD assessment in academic studies[12,23,26], the calculation of Clermont score is based on quantitative ADC values and wall thickness along with qualitative signs of bowel ulcer and mural edema. As a completed quantitative analysis of DWI in this study, Clermont score was not applied for its qualitative signs on conventional MRE. And the Kappa values of inter-reader agreement were only fair for the presence of ulcer (0.34) and moderate for the presence of mural edema (0.47) on conventional MRE[30]. Furthermore, ADC values were reported to have similarly high diagnostic performance for CD patients compared with Clermont score in prior studies[9,23]. In addition, ADC measurement from the post-processing ADC maps and SR of the terminal ileum to ipsilateral psoas muscle on b = 800 s/mm^2 images are relatively convenient to implement in the daily clinical practice.

However, the measured ADC in normal terminal ileum demonstrated lower agreement between sessions and readers (ICC: 0.641-0.736; CoV: 10.47%-11.43%; BA-LA LoA: ±26.59% to ±30.83%). This might be due to more pronounced partial volume effects of the thin normal intestinal tissues during the ROI placement compared to the thickened inflammatory segments[5]. Additionally, post-processing ADC maps are generated by applying a mono-exponential fit with at least two b values, a technique prone to image degradation due to bowel peristalsis[9,31]. The SR in the subjects with a normal terminal ileum still achieved high intra and interobserver agreements (ICC:

Table 3 Interobserver agreement and variability of apparent diffusion coefficient and signal ratio measurements between the two readers over two sessions

Group	CD (*n* = 21)						Normal (*n* = 23)					
	1st session reader 1	1st session reader 2	*P*-value	2nd session reader 1	2nd session reader 2	*P*-value	1st session reader 1	1st session reader 2	*P*-value	2nd session reader 1	2nd session reader 2	*P*-value
ADC	1.50 ± 0.40	1.52 ± 0.45	0.402	1.54 ± 0.45	1.56 ± 0.47	0.379	2.55 ± 0.53	2.57 ± 0.50	0.815	2.53 ± 0.53	2.64 ± 0.57	0.208
ICC	0.969			0.984			0.641			0.736		
CoV	4.71			3.73			11.43			11.07		
Bias (%)	-0.72			-0.82			-1.20			-3.98		
LoA (%)	-14.29 to 12.85			-12.09 to 10.44			-32.03 to 29.62			-33.93 to 25.96		
SR	4.17 ± 1.43	4.21 ± 1.34	0.473	4.20 ± 1.36	4.23 ± 1.42	0.569	1.45 ± 0.54	1.44 ± 0.52	0.760	1.44 ± 0.53	1.45 ± 0.56	0.618
ICC	0.981			0.989			0.950			0.971		
CoV	4.53			3.72			8.12			6.27		
Bias (%)	-1.85			-0.39			0.43			-0.59		
LoA (%)	-17.30 to 13.60			-12.38 to 11.61			-22.17 to 23.04			-19.47 to 18.29		

Apparent diffusion coefficient (ADC) and signal ratio parameters are expressed as the mean ± standard deviation, ADC units: $\times 10^{-3}$ mm^2/s. ADC: Apparent diffusion coefficient; SR: Signal ratio; CD: Crohn's disease; ICC: Intraclass correlation coefficient; CoV: Coefficients of variation; LoA: Bland-Altman analysis with 95% limits of agreement.

0.944-0.974) and was less variable than ADC between sessions and readers. The main reasons could be that SR is calculated from a single b value DWI, limiting the propensity for motion degradation, and acquisition of a single b value DWI image using SS-EPI is rapid, sub-second using the latest acquisition techniques and hardware[8,20]. Hence, SR could be more robust for quantitative DWI assessment in normal or inflamed ileal segments of CD patients. And the measurement of SR avoids the post-processing maps compared to ADC, which would be easier and more applicable in clinic.

In line with prior quantitative analysis of DWI on CD patients[9,23,29], the ADC values of inflammatory lesions were significant lower than those of normal segments in this study, and achieved optimal AUROC (R1: 0.923 and R2: 0.929) with high sensitivities and specificities by a threshold value of 1.8 × 10^{-3} mm^2/s. Furthermore, diagnostic efficiency of SR (AUROC: 0.981 for R1 and 0.988 for R2) was slightly superior to the ADC values. According to our results and Table 4, SR values greater than 2 (meaning more than twice signal intensity of the terminal ileum to ipsilateral psoas muscle) could be used to differentiate inflamed terminal ileum from the normal. Quantitative parameters of ADC and SR from DWI both had excellent diagnostic accuracy for CD. Similarly, the relative signal intensity (rSI) of lesion to muscle demonstrated higher diagnostic performance than ADC in assessing complete tumor response on post neoadjuvant chemoradiotherapy DWI in rectal cancer[24]. The SR, as an alternative modality of ADC, would be a reliable and effective quantitative parameter of DW-MRE to identify the normal and inflamed bowel segments based on this initial study. The further exploration of SR in the differentiation of CD stages and medical therapy assessment during CD course is worthwhile in the future study.

However, our study had some limitations due to the retrospective nature and relatively small sample size included. Further prospective trials with larger sample sizes would help to confirm these results. Since DW-MRE examinations of this primary study were acquired from a single center using a same 3.0-T MR scanner, a multicenter study with various MR systems could further increase the applicability of the data. Additionally, ROIs placement on the bowel wall was relatively challenging and subjective for the observers; improvements in automated approaches to ROI placement would be potentially of great value. Finally, the quantitative endoscopic score was not acquired due to this retrospective study.

In conclusion, the intra and interobserver agreements of quantitative ADC and SR

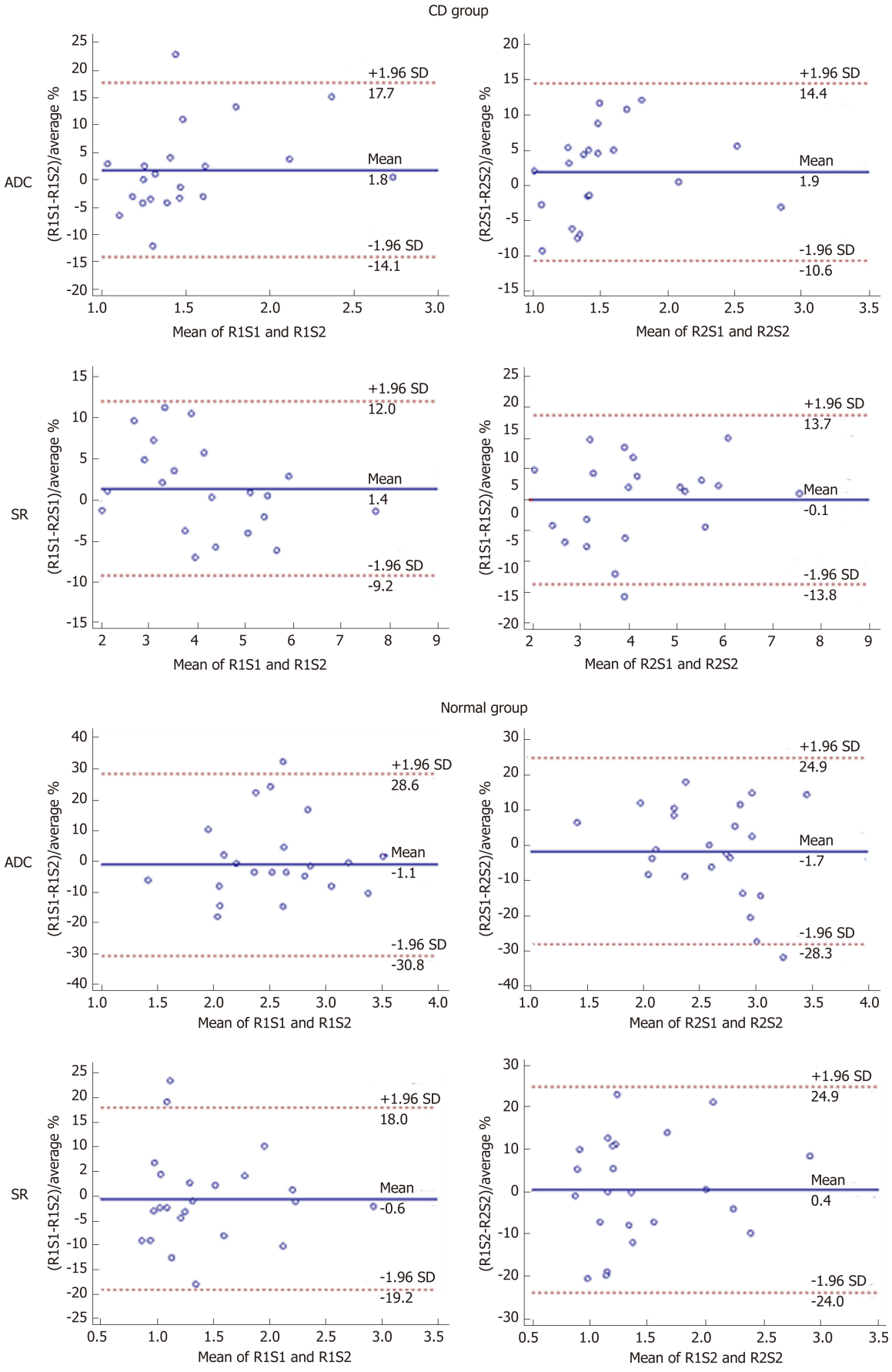

Figure 3 Bland-Altman plots of intraobserver agreement between 2 two sessions of two readers. Bland-Altman 95% limits of agreement with diffusion weighted imaging parameters including apparent diffusion coefficient and signal ratio from the Crohn's disease group and normal group. The solid line represents the mean relative difference as a percentage and the dashed lines represent the upper and lower 95% limits of agreement. ADC: Apparent diffusion coefficient; SR: Signal ratio; CD: Crohn's disease; SD: Standard deviation; R1S1: First session of reader 1; R1S2: Second session of reader 1; R1S2: First session of reader 2; R2S2: Second session of reader 2.

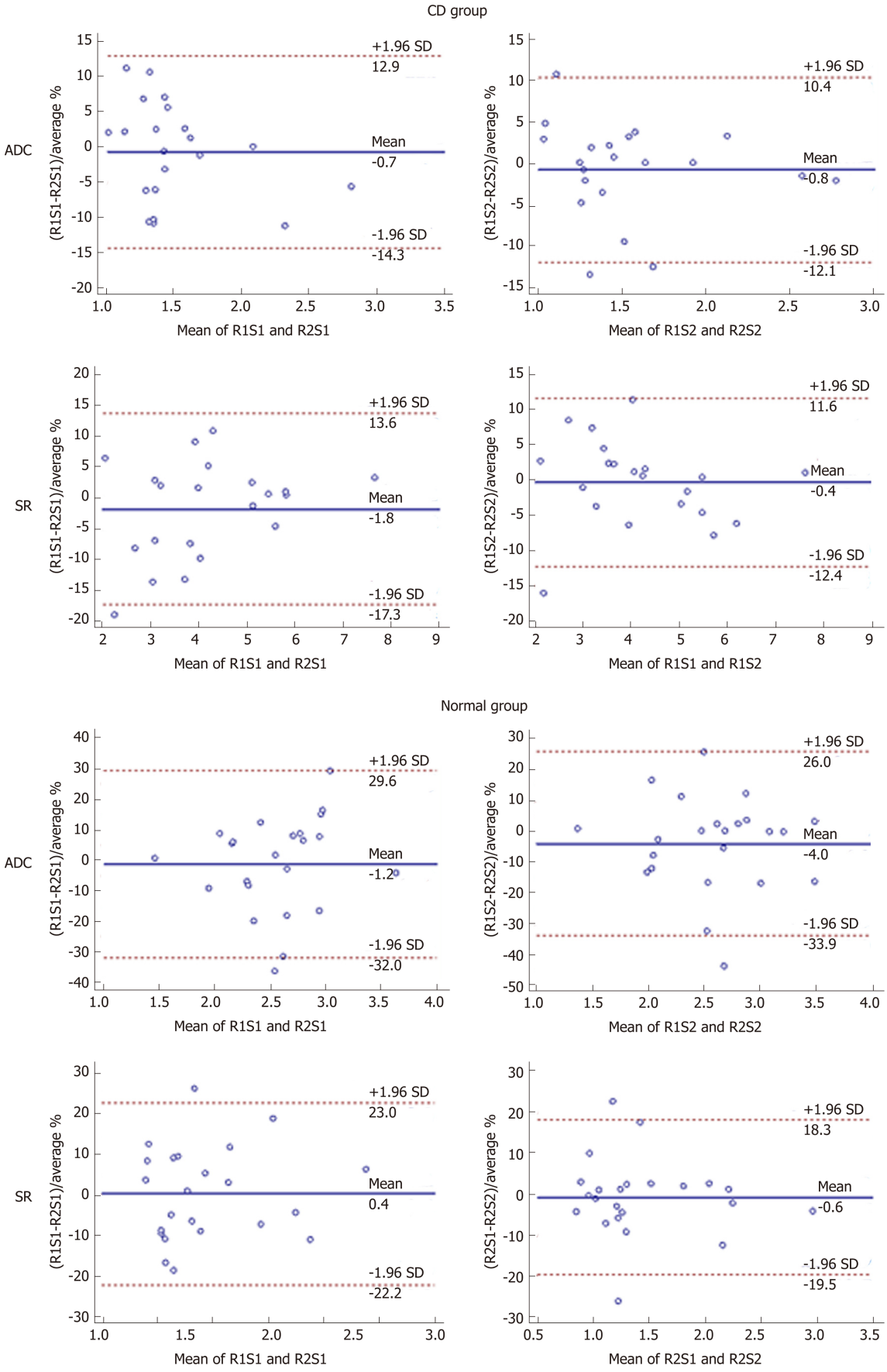

Figure 4 Bland-Altman plots of interobserver agreement between two readers at two sessions. Bland-Altman 95% limits of agreement with Crohn's disease parameters including apparent diffusion coefficient and signal ratio from the Crohn's disease group and normal group. The solid line represents the mean relative difference as a percentage and the dashed lines represent the upper and lower 95% limits of agreement. ADC: Apparent diffusion coefficient; SR: Signal ratio; CD: Crohn's disease; SD: Standard deviation; R1S1: First session of reader 1; R1S2: Second session of reader 1; R1S2: First session of reader 2; R2S2: Second session of reader 2.

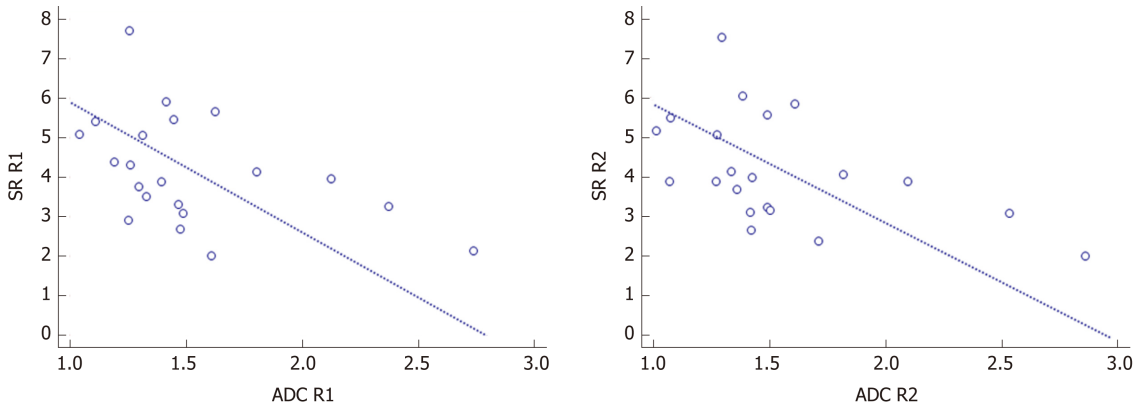

Figure 5 Scatterplots between apparent diffusion coefficient and signal ratio of reader 1 and reader 2. Reader 1: r = -0.438, P = 0.047; Reader 2: r = -0.485, P = 0.026; R1: reader 1; R2: reader 2. ADC: Apparent diffusion coefficient; SR: Signal ratio; R1: Reader 1; R2: Reader 2.

analysis on DW-MRE images were found to be good or excellent in this initial study. Both the ADC and SR demonstrated high diagnostic yield for distinguishing inflamed terminal ileum in CD patients from the normal. Therefore, ADC value or SR could serve as robust and efficient biomarkers to aid in the noninvasive evaluation of CD.

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Table 4 Diagnostic performance of apparent diffusion coefficient and signal ratio for differentiating inflammatory lesions from normal terminal ileum

	CD (*n* = 21)	Normal (*n* = 23)	*P*-value	Cut-off value	AUROC	Sensitivity (%)	Specificity (%)
First reader							
ADC value	1.52 ± 0.42	2.54 ± 0.49	< 0.001[a]	1.80	0.923	85.7	95.7
SR value	4.19 ± 1.39	1.44 ± 0.53	< 0.001[a]	2.24	0.981	90.5	95.7
Second reader							
ADC value	1.54 ± 0.46	2.61 ± 0.48	< 0.001[a]	1.81	0.929	85.7	95.7
SR value	4.22 ± 1.37	1.45 ± 0.53	< 0.001[a]	2.07	0.988	95.2	95.7

[a]$P < 0.05$ was considered statistically significant. Apparent diffusion coefficient (ADC) and signal ratio parameters are expressed as the mean ± SD, ADC units: $\times 10^{-3}$ mm^2/s. ADC: Apparent diffusion coefficient; SR: Signal ratio; CD: Crohn's disease; AUROC: Areas under receiver operating characteristic curve.

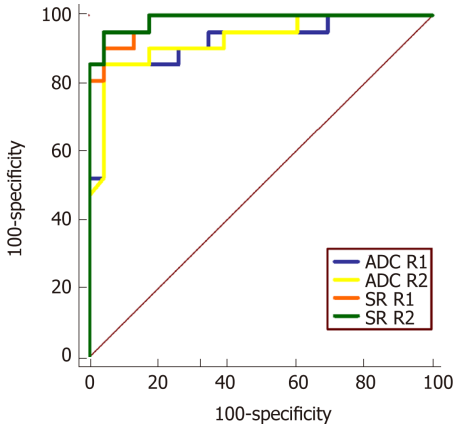

Figure 6 Receiver operating characteristic curve for quantitative apparent diffusion coefficient and signal ratio of the two readers. ADC: Apparent diffusion coefficient; SR: Signal ratio; R1: Reader 1; R2: Reader 2.

ARTICLE HIGHLIGHTS

Research background

The diagnosis and monitoring of Crohn's disease (CD) are important for the clinical therapy during its longstanding course. As avoiding the use of intravenous contrast agents, diffusion weighted (DW) imaging (DWI) is increasingly considered as a promising magnetic resonance (MR) enterography (MRE) technique to the qualitative and quantitative evaluation of CD in recent years, and is recommended as an optional MR sequence for CD. The quantitative DWI analysis may be more objective for CD assessment. However, the measurement reproducibility of quantitative DWI analysis in MRE has not been ascertained so far. Thus, the investigation of intra and interobserver agreements of quantitative DWI parameters could be valuable to facilitate the application of quantitative DW-MRE into the clinical routine.

Research motivation

DWI has shown a superb potential as a quantitative modality for CD evaluation. The characterization of reader agreement and variability with quantitative DWI parameters is imperative before such parameters are to be incorporated into clinical practice. Therefore, it is necessary to investigate the level of measurement reproducibility of quantitative DWI parameters.

Research objectives

This study aimed to explore intra and interobserver reproducibility of quantitative analysis for DW-MRE in CD, and then to assess the diagnosis efficiency of quantitative parameters for the evaluation of CD.

Research methods

Forty-four subjects (21 diagnosed with CD and 23 normal control subjects) who underwent ileocolonoscopy and DW-MRE (b = 800 s/mm^2) within one week were included. Two radiologists, blinded to clinical details as well as endoscopy results, measured the signal intensity (SI) of the terminal ileum and ipsilateral psoas muscle on DWI with b = 800 s/mm^2. Separately and simultaneously, apparent diffusion coefficients (ADC) of the terminal ileum were

recorded. Then, SI of ipsilateral psoas muscle on DWI with b = 800 s/mm^2 was also measured by placing a larger oval ROI. Signal intensity ratio (SR) of the terminal ileum to ipsilateral psoas muscle was calculated according to the equation: $SR = SI_{ileum}/SI_{muscle}$. After a washout period of 4 weeks, the same two radiologists repeated the above described measurements to assess intraobserver agreement. Between- and within-reader agreements were assessed using intraclass correlation coefficients (ICC), coefficients of variation (CoV), and 95% limits of agreement of Bland-Altman plots (BA-LA LoA). Diagnostic performances of ADC and SR for identifying inflamed terminal ileum from the normal were evaluated by receiver operating characteristic (ROC) curve analysis.

Research results

There were no significant differences in quantitative DWI parameters (ADC and SR) between the two sessions or between the two radiologists either in the CD or control group (paired *t*-test, $P > 0.05$). The intra and interobserver reproducibility of ADC (ICC: 0.952-0.984; CoV: 3.73%-6.28%; BA-LA LoA: ±11.27% to ±15.88%) and SR (ICC: 0.969-0.989; CoV: 3.51%-4.64%; BA-LA LoA: ±10.62% to ±15.45%) was excellent for CD. Agreement of ADC measurements was slightly less in control subjects (ICC: 0.641-0.736; CoV: 10.47%-11.43%; BA-LA LoA: ± 26.59% to ±30.83%). SR of the normal terminal ileum demonstrated high intra and interobserver reproducibility (ICC: 0.944-0.974; CoV: 3.73%-6.28%; BA-LA LoA: ± 18.58% to ± 24.43%). The areas under the ROC curve for SR (reader 1: 0.981; reader 2: 0.988) were slightly better than those of ADC (reader 1: 0.923; reader 2: 0.929), but these differences were not statistically significant (*P*-values: 0.117 for reader 1; 0.067 for reader 2). The cutoff values maximizing sensitivity and specificity for ADC and SR parameters were approximately 1.80 × 10^{-3} mm^2/s and 2.00, respectively. In addition, SR was moderately and negatively correlated with ADC values in CD patients for both readers (reader 1: $r = -0.438$, $P = 0.047$; reader 2: $r = -0.485$, $P = 0.026$).

Research conclusions

From this study, the intra and interobserver agreements of quantitative ADC and SR analysis from DW-MRE images were found to be good or excellent. Both the ADC and SR demonstrated high diagnostic yield for distinguishing inflamed terminal ileum in CD patients from the normal. Additionly, SR demonstrated moderately negative correlations with ADC in CD patients, and could also represent the diffusion condition of the terminal ileum. Therefore, ADC value or SR could serve as robust and efficient biomarkers to aid in the noninvasive evaluation of CD.

Research perspectives

Quantitative ADC and SR values derived from DW-MRE achieved good to excellent intra and interobserver agreements with high diagnostic accuracy based on this initial study. The further exploration of these quantitative parameters in the differentiation of CD stages and medical therapy assessment during CD course is worthwhile in the future study.

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SYSTEMATIC REVIEWS

Bioartificial liver support systems for acute liver failure: A systematic review and meta-analysis of the clinical and preclinical literature

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Abstract

BACKGROUND

Acute liver failure (ALF) has a high mortality varying from 80% to 85% with rapid progress in multi-organ system failure. Bioartificial liver (BAL) support systems have the potential to provide temporary support to bridge patients with ALF to liver transplantation or spontaneous recovery. In the past decades, several BAL support systems have been conducted in clinical trials. More recently, concerns have been raised on the renovation of high-quality cell sources and configuration of BAL support systems to provide more benefits to ALF models in preclinical experiments.

AIM

To investigate the characteristics of studies about BAL support systems for ALF, and to evaluate their effects on mortality.

METHODS

Eligible clinical trials and preclinical experiments on large animals were identified on Cochrane Library, PubMed, and EMbase up to March 6, 2019. Two reviewers independently extracted the necessary information, including key BAL indicators, survival and indicating outcomes, and adverse events during treatment. Descriptive analysis was used to identify the characteristics of the included studies, and a meta-analysis including only randomized controlled trial

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(RCT) studies was done to calculate the overall effect of BAL on mortality among humans and large animals, respectively.

RESULTS
Of the 30 selected studies, 18 were clinical trials and 12 were preclinical experiments. The meta-analysis result suggested that BAL might reduce mortality in ALF in large animals, probably due to the recent improvement of BAL, including the type, cell source, cell mass, and bioreactor, but seemed ineffective for humans [BAL *vs* control: relative risk (95% confidence interval), 0.27 (0.12-0.62) for animals and 0.72 (0.48-1.08) for humans]. Liver and renal functions, hematologic and coagulative parameters, encephalopathy index, and neurological indicators seemed to improve after BAL, with neither meaningful adverse events nor porcine endogenous retrovirus infection.

CONCLUSION
BAL may reduce the mortality of ALF by bridging the gap between preclinical experiments and clinical trials. Clinical trials using improved BAL must be designed scientifically and conducted in the future to provide evidence for transformation.

Key words: Bioartificial liver; Acute liver failure; Preclinical experiment; Clinical trial; Meta-analysis

Core tip: This systematic review and meta-analysis included a large number of studies about clinical trials and preclinical experiments of bioartificial liver (BAL) support systems for treating patients and large animal models with acute liver failure. We summarized the characteristics of studies, BAL, and outcomes in all the studies and compared the pooled effect by meta-analysis including only randomized controlled trial studies regarding mortality after BAL among humans and large animals, respectively.

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INTRODUCTION

Acute liver failure (ALF) is characterized by an acute episode of liver dysfunction in individuals without underlying chronic liver diseases, sometimes causing a rapid onset of encephalopathy and coagulopathy followed by multiorgan system failure. Patients with ALF have a high mortality ranging from 80% to 85%, approaching 90% among those with severe fulminant hepatic failure (FHF)[1]. The most effective treatment method for patients with ALF is liver transplantation, as it has increased the 5-year survival rate by 75%[2]. Although some patients might recover spontaneously, many would die during waiting for a compatible donor because of aggressive deterioration of liver function or development of cerebral edema, intracranial hypertension, and even irreversible brain damage. Thus, a liver support system must be developed to maintain a viable status of these patients prior to the transplantation.

During the past decades, several artificial devices for removing toxins from patients' blood through filtration and adsorption have improved clinical status in some cases. However, a meta-analysis of six randomized controlled trials (RCTs) concluded that artificial liver support systems might not reduce the mortality in ALF[3]. Moreover, the newly developed bioartificial liver (BAL) support systems that incorporate a hepatoma cell line or primary hepatocytes into a bioreactor when processing blood or plasma proved meaningful for prolonging the survival time of ALF animals in preclinical trials. Several types have been applied for the treatment of patients with ALF in phase I studies or controlled clinical trials, and improved neurological status and liver and renal functions, thus bridging to transplantation or

spontaneous recovery[4-7]. However, the survival outcome and adverse effects of such alternative methods are controversial[3,8].

In addition, the BAL has various types with different cell sources, cell mass, and culture methods, as well as architectural design such as the bioreactor, scaffold, and separation, which might be associated with the effect and safety of the BAL in treating ALF[9]. Furthermore, the BAL has been modified and renovated in preclinical experiments on large animals but has not been used in clinical trials[10-12], which caused a significant gap between clinical and preclinical studies.

One objective of our study was to investigate the characteristics of studies about BAL for ALF in both clinical trials and recent preclinical experiments on large animals. In particular, we looked at key indicators of the BAL, survival outcome, and adverse events regarding the treatment. Another objective was to evaluate the pooled effect of the BAL on mortality by conducting a meta-analysis of randomized controlled studies stratified among patients with ALF and large animals.

MATERIALS AND METHODS

This study was constructed following the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines. The protocol has been registered in PROSPERO, an international prospective register of systematic reviews (Registration number: CRD42019133215).

Inclusion and exclusion criteria

We included studies about any of the BAL for ALF, including all clinical trials, case reports, and RCTs in patients with ALF and preclinical experiments in large animals (monkeys, pigs, and dogs) published in the past 10 years. The language was limited to English.

The exclusion criteria were as follows: (1) Not focusing on the outcome of BAL, or the ALF group could not be separated from the other study populations such as patients with acute-on-chronic liver failure; (2) Duplicates of previous publications; (3) Based on incomplete data; and (4) Reviews, meta-analyses, comments, guidelines, letters, editorial articles, and project or conference summaries. If more than one study by the same author using the same data was published, either the study with the largest sample size or the most recently published study was included.

Literature search and selection

By using a searching strategy and filter that combined keywords or subjects about BAL and ALF, which had been pre-tested and improved repeatedly, we searched the Cochrane Library, PubMed, and EMbase to identify eligible articles till March 6, 2019 according to the inclusion and exclusion criteria, by setting the following key elements (Patients: ALF; Intervention: BAL; Comparison: None; Outcome: including but not limited to mortality, bridging time, liver and renal function; keywords used for literature search are shown in Supplementary material). Literature about preclinical experiments in large animals was limited to papers published in the past 10 years. The references used in the eligible articles were also reviewed to examine other potential sources.

Teams of paired reviewers who were trained and knowledgeable about the study screened the literature independently. We screened the title and keywords first and excluded unqualified studies according to the predefined criteria. Then, we read the abstracts and full texts carefully to further exclude unqualified literature. The decision to exclude studies was determined by two reviewers. Inconsistent results were resolved either by discussion or decided by a third reviewer. Finally, the remaining studies were enrolled to be reviewed and analyzed. The flow chart of the study selection is shown in Figure 1.

Data extraction

We extracted the following information from each eligible article: (1) Basic information of the included studies, including the publication year, title of the article, journal along with impact factor in 2018, country of the first author, study setting, study type (clinical trial, case report, or preclinical experiment), and data sources (full text and abstract); (2) Detailed information of clinical trials in humans, including the type of BAL [*e.g.*, HepatAssist, extracorporeal liver assist device (ELAD), academic medical center (AMC)-BAL, modular extracorporeal liver support (MELS), novel bioartificial liver support system (BLSS), radial-flow bioreactor (RFB)-BAL, and hybrid bioartificial liver (HBAL)], whether hybrid or not, cell sources (*e.g.*, porcine hepatocytes, C3A cells, or primary human hepatocytes), cell mass, sample size, ALF subtype [fulminant hepatic failure (FHF) or primary nonfunction (PNF)], age and sex

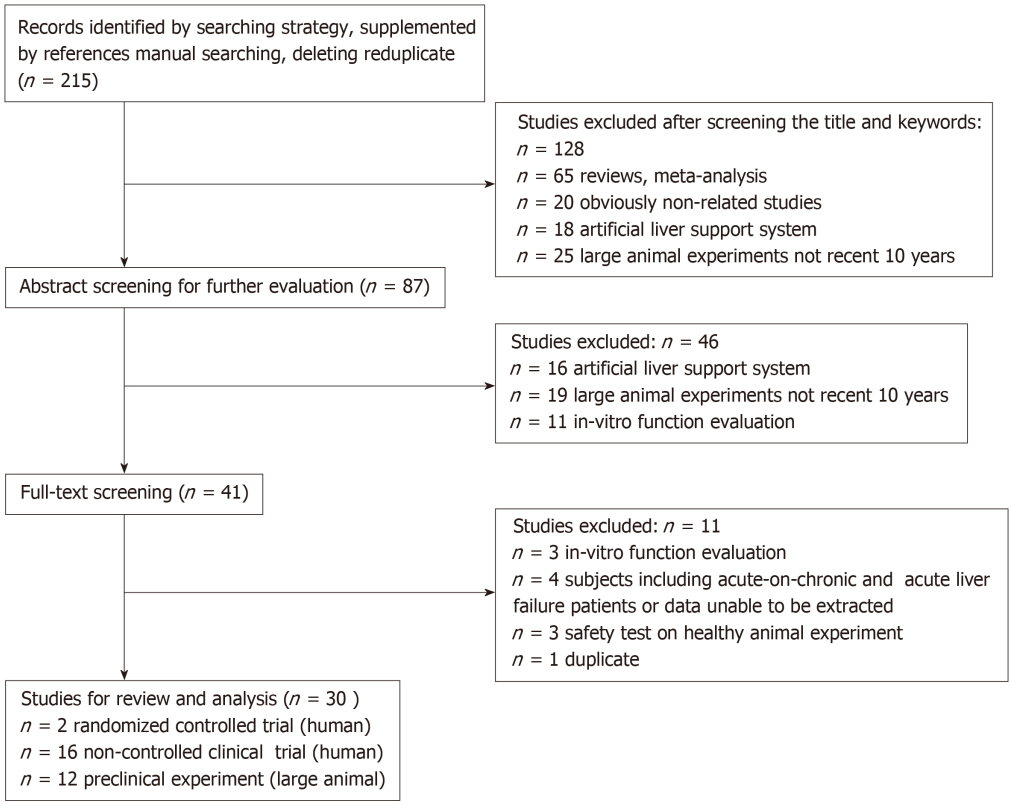

Figure 1 Flow chart of study selection.

of subjects, disease etiology [*e.g.*, PNF, viral, indeterminate, autoimmune, Acetaminophen (AO), and ischemic], BAL treatment time, outcomes (*i.e.*, bridging time, orthotopic liver transplantation, death events, and recovery), follow-up time, stable or unstable hemodynamics, effects on liver and renal functions, hematologic and coagulative parameters, encephalopathy index, neurological status, adverse events during treatment (*e.g.*, transitory hypotension), and porcine endogenous retrovirus (PERV) test result; (3) data of preclinical experiment on large animals, including animal species (*e.g.*, pig, canine, and monkey), number of animals, sex (female and male), weight (kg), inducer of acute liver failure (*e.g.*, D-galactosamine, hepatic artery ligation, surgical ligation of all blood flow to the liver, α-amanitin, and lipopolysaccharide), type of BAL (*e.g.*, FBBAL, HBAL, HBALSS, SRBAL, hiHep-BAL, and UCLBAL), cell sources (*e.g.*, alginate-chitosan encapsulated primary porcine hepatocytes, co-cultured pig hepatocytes, bone marrow mesenchymal stem cells, human hepatic CL-1 cells grown in microgravity culture, HiHeps, and three-dimensional HepG2-cell spheroids), cell mass, bioreactor (*e.g.*, choanoid fluidized bed bioreactor, multi-layer flat-plate bioreactor anionic resin adsorption column, fluidized-bed bioreactor, perfusion bioreactor, spheroid reservoir, and packed-bed bioreactor), treatment time, survival time or rate at a specific time point, other effects such as ammonia level, and PERV test result.

For data extraction and scoring, paired reviewers conducted the survey independently on the basis of the literature database and recorded the necessary information. The results of the paired reviewers were cross-checked, and disagreements were resolved by discussion or decided by a third reviewer.

Statistical analysis

We conducted description analyses of the study characteristics of clinical trials and preclinical experiments on large animals by using the absolute numbers and percentages of the qualitative variables, and mean [standard deviation (SD)] or median (percentile) of the quantitative data. The bias risk of the included RCT studies was assessed according to the Cochrane assessment method for RCTs, while risk of non-RCT studies was assessed according to the Newcastle-Ottawa Quality Assessment Scale for cohort studies.

By selecting clinical RCTs and preclinical RCT experiments with survival outcome (death event) recorded at a specific time, we combined the effect of selected studies on the relative ratio (RR) scale by performing a meta-analysis with a random effect model

and assigning weights according to the estimated variance. The heterogeneity of the included studies was also tested, with Q-test significance ($P < 0.05$) or $I2 > 50\%$ indicating that heterogeneity existed between studies. The overall RR and 95% confidence interval (CI) were calculated. We also conducted sensitivity analyses to examine the impact of using alternative effect measures (odds ratio *vs* relative ratio), pooling methods [Peto *vs* Mantel-Hanszel (M-H)], and statistical models (fixed- *vs* random-effects). Begg's funnel plot was also used to evaluate the publication bias.

All the statistical analyses were performed with SPSS 23.0 (IBM Corp. Armonk, NY, United States) and Review Manager 5.3 (RevMan, the Cochrane Collaboration, Oxford, England).

RESULTS

We identified 215 studies preliminarily, and then excluded 67 unrelated or ineligible articles, 34 articles about artificial liver support system, 14 articles about *in vitro* function evaluation, 1 duplicate article, 4 articles with unavailable data, and 65 reviews. We finally included 30 articles in the analyses, of which 18 were human clinical trials[6,7,13-28] (only 2 were RCT studies[6,7]) and 12 were preclinical experiments in large animals[5,10-12,29-36].

Among the 30 articles, 94.4% (17/18) were clinical trials published before 2005, with only 1 phase I clinical trial published in 2018 (only abstract available). Studies about preclinical experiments in large animals in the recent 10 years accounted for 40% of the articles (12/30). The median (P_{25}-P_{75}) impact factor (IF) of the journals was 4.04 (2.60-9.20), with 8 articles having an IF < 3 and 5 having an IF > 10. Approximately two-thirds of the studies were conducted by authors in the United States (11/30) and China (8/30). One RCT study was done in England and published in *Hepatology* in 1996[7] while the other one was performed in 11 United States and 9 European sites by United States researchers and published in *Annals of Surgery* in 2004[6]. More than one-third (12/30) of the studies were about hybrid support systems (*e.g.*, HBAL, MELS, HBALSS, and HepatAssist), distributed in 9 clinical trial studies and 3 preclinical experimental studies. The available data that we analyzed were mostly from full texts (29/30), and 1 abstract of clinical trial was also included considering the limited number of eligible studies in humans and the availability of valuable information in the abstract (as shown in Supplemental Table 1).

Characteristics of the BAL, subjects, outcomes, and adverse events in the clinical trials

From the 18 clinical trials, 332 patients with ALF were included, with 295 cases of FHF subtypes and 37 cases of PNF subtypes. The mean age was 35.4 years, and females accounted for 69.4% of the patients according to the reported data. Most of the disease etiologies were indeterminate (103 cases), viruses (45 cases), and AO (33 cases). The types of BAL included HepatAssist (6 cases), ELAD (3 cases), AMC-BAL (2 cases), MELS (2 cases), BLSS (1 case), RFB-BAL (1 case), HBAL (1 case), Lifeliver (1 case), and BAL (1 case), and in one case the BAL type was unclear; of these 9 were hybrids and 8 were non-hybrids. Most of the cells were sourced from porcine hepatocytes (14/18), followed by C3A cells (3/18) and primary human hepatocytes (1/18). The mean cell mass was approximately 9×10^9 (Table 1).

The mean treatment time was around 25 h. Among the 317 reported patients, 201 received orthotopic liver transplant (OLT) and 57 recovered without OLT. The mean survival rate in the OLT group was 93%, with the follow-up period ranging from 7 d to 62 mo. Hemodynamics were stable in all patients. These results show that BAL improved liver and renal functions in all the patients, except 3 with PNF[6,14,15], in terms of the different biochemical parameters, with decreased ammonia, bilirubin, and transaminase levels in 12, 12, and 10 studies, respectively. Other meaningful indicators were reduced, including lactate ALB, BUN, and creatinine levels. By examining prothrombin time (PT), international normalized ratio, or other parameters, 9 studies found improvements in hematological and coagulative status. Ten of 12 reported studies showed an improvement in encephalopathy index. Thirteen of 16 reported studies showed improvements in neurological indicators, showing decreased intracranial pressure (ICP) and increased cerebral perfusion pressure, Glasgow coma score, and comprehensive level of consciousness score. In 3 studies on ALF subtypes, the encephalopathy index and neurological indicators showed improvements in patients with FHF but not in patients with PNF[6,14,15]. However, one case report of a patient with PNF showed improvement in the neurological status with a change in coma stage from IV to I after BAL treatment[22] (Table 2).

Table 1 Characteristics of bioartificial liver support system and subjects of clinical trials

ID	Publication year	Type of trial	BAL system				Subject				
			Type	Hybrid	Cell	Mass	Sample No.	Subtype	Age	Sex	Disease etiology
1	1994	Non-controlled trial	HepatAssist	Yes	Porcine hepatocytes	6×10^9	7	FHF 6 PNF 1	10-58	4M:3F	PNF 1 ; Virus 1; AO 2; Indeterminate 3
2	1997	Non-controlled trial	HepatAssist	Yes	Porcine hepatocytes	5×10^9	21	FHF 18 PNF 3	36.1 ± 3.4 48.3 ± 11.2	7M:11F 1M:2F	Virus 4; Indeterminate 8; AO 4; Ischemic 2 Virus 1; Indeterminate 1; Autoimmune 1
3	2010	Non-controlled trial	HepatAssist	Yes	Porcine hepatocytes	5×10^9	18	FHF 15 PNF 3	10-56 26-58	10M;5F 1M:2F	Indeterminate 7; Virus 3; AO 4; Ischemic 1 Indeterminate 1; Virus 1; Autoimmune 1;
4	2002	Non-controlled trial	HepatAssist	Yes	Porcine hepatocytes	5×10^9	10	FHF 10	31	4M:6F	Indeterminate 6; Virus 2; Wilson 1; Pyrazinamide 1
5	1994	Non-controlled trial	ELAD	No	C3A cells	200 g	11	FHF 10 PNF 1	38.5 ± 18.1	5M:6F	Idiopathic 1; Virus 3; AO 2; INH 1; FIAU 3; Anhepatic 1
6	2002	Non-controlled trial	ELAD	No	C3A cells	300-400g	5	FHF 5	22.2 ± 9.4	2M:3F	L-asparaginase toxicity 1; Indeterminate 3; Autoimmune 1
7	2002	Phase I trial	AMC-BAL	No	Porcine hepatocytes	11.9×10^9	7	FHF 7	34.3 ± 15.2	2M:5F	Virus 4; AFLP 1; Indeterminate 2
8	2003	Case report	AMC-BAL	No	Porcine hepatocytes	10×10^9	1	FHF	35	F	Virus 1
9	1996	Non-controlled trial	NR	No	Porcine hepatocytes	5×10^9	12	FHF 11 PNF 1	37.2 ± 15.5	6M:6F	Indeterminate 4; AO 2; Virus 3; Ischemic Failure 1; Autoimmune 1; PNF 1
10	2003	Phase I trial	MELS	Yes	Porcine hepatocytes; non-parenchymal cells	$(1.8\text{-}4.4) \times 10^{10}$	8	FHF 8	34.3	1M:7F	Rug-related 2; Virus 3; Indeterminate 3
11	2003	Case report	MELS	Yes	Human hepatocytes	470 g	1	PNF	26	F	Intoxication with amanita phalloides
12	2002	Case report	BLSS	No	Porcine hepatocytes	70-100 g	1	FHF	41	F	Indeterminate 1
13	2002	Non-controlled trial	RFB-BAL	No	Porcine hepatocytes	200-230 g	7	FHF 4 PNF 3	37.4 ± 18.4	5M:2F	Virus 3; PNF 3; Liver trauma 1
14	2003	Non-controlled trial	HBAL	Yes	Porcine hepatocytes	1.0×10^9	12	FHF 12	41.8 ± 13.0	9M:3F	Virus 12
15	1999	Non-controlled trial	HepatAssis	Yes	Porcine hepatocytes	5×10^9	8	FHF 8	33.4 ± 11.0	1M:7F	AO 8
16[1]	2018	Phase 1/2a trial	Lifeliver BAL	NR	Porcine hepatocytes spheroids	NR	8	FHF 8	NR	NR	NR
17	1996	Pilot-Controlled trial	ELAD	No	C3A cells	400 g	Group-1 17 Group-2 7	FHF 17 FHF 7	30	6M:3F *vs* 3M:5F[2] 0M:3F *vs* 3M:1F[2]	AO 5, Virus 3, ATB 5 *vs* AO 6, Virus 1[2]; ATB 1 AO 3 *vs* AO 3, Virus 1[2]
18	2004	RCT	HepatAssist	Yes	Porcine hepatocytes	7×10^9	171	FHF 147 PNF 24	37	26M:60F *vs* 25M:60F[2]	Known causes 83, Indeterminate 64

[1]Data from abstract;

[2]Intervention *vs* control. NA: Not applicable; NR: Not reported; Sex: Male (M) and Female (F); ELAD: Extracorporeal liver assist device; BAL: Bioartificial liver; AMC-BAL: Academic medical center bioartificial liver; MELS: Modular extracorporeal liver support; BLSS: Novel bioartificial liver support system; RFB-BAL: Radial-flow bioreactor bioartificial liver; HBAL: Hybrid bioartificial liver; FBBAL: Fluidized bed bioartificial liver; HBALSS: Hybrid bioartificial liver support system; PNF: Primary nonfunction; FHF: Fulminant hepatic failure; AO: Acetaminophen; INH: Isoniazid; FIAU: Fialuridine; AFLP: Acute fatty liver of pregnancy; ATB: Anti-tuberculous hepatotoxicity; PERV: Porcine endogenous retrovirus; RCT: Randomized controlled trial.

During BAL treatment, 9 studies reported adverse events such as transient hypotension, decreased body temperature, tachycardia, pyrexia, and hypoglycemia, which had no clinical significance and resolved in all. PERV test results in 8 reported studies were all negative (Table 2).

Characteristics of the BAL, animal, and outcomes in the preclinical experiments with large animals

Of 12 studies that performed preclinical experiments with pigs (8 studies, 160 animals), monkeys (2 studies, 45 animals), and canines (2 studies, 40 animals), the proportions of male and female animals were 45.3% (111/245) and 30.2% (74/245), respectively. Inducers included D-galactosamine in 7 studies (145 animals)[5,12,29-33], surgicaloperation in 3 studies (53 animals)[11,34,35], 85% hepatectomy in 1 study (18 animals)[36], and α-amanitin and lipopolysaccharide in 1 study (30 animals)[10] (Table 3).

The types of BAL included SRBAL (n = 3), HBAL (n = 2), FBBAL (n = 1), UCLBAL (n = 1), hiHep-BAL (n = 1), HBALSS (n = 1), FBBAL (n = 1), and BAL (n = 1), using cells from porcine hepatocyte (9/12), human hepatic CL-1 cells or HepG2 cells (2/12), and HiHeps (1/12), which were all cultured using modified three-dimensional methods such as spheroids, organoid, alginate-chitosan encapsulated, and even microgravity culture, with corresponding bioreactors such as a spheroid reservoir. The mean cell mass was 2×10^{10}, and the treatment time ranged from 3 to 24 h (mean: 9 h; Table 3).

Compared with the control group, the survival outcomes (median survival time or survival rate) were better in the BAL group, and the biochemical metabolic function showed improvement, especially decreased ammonia levels (10/12), bilirubin levels (6/12), ICP (5/12), and PT (4/12). All the PERV test results were negative in reported studies (Table 3).

Meta-analysis of the effects of the BAL on mortality in the clinical trials and preclinical experiments

As shown in Figure 2, the overall effect of the BAL on mortality in the patients with ALF[6,7] was insignificant [BAL 97 *vs* control 98: RR (95%CI), 0.72 (0.48-1.08)]. However, the meta-analysis of the preclinical experiments of large animals[10,12,29,30,32,36] indicated a significant effect of the BAL [BAL 56 *vs* control 77: RR (95%CI), 0.27 (0.12-0.62)]. The test of heterogeneity showed no significant difference between included studies, with I^2 being 0% and 41% for human clinical studies and pre-clinical experimental studies, respectively.

Sensitivity analyses of pooled results using an alternative effect measure (M-H OR = 0.61, 95%CI: 0.33-1.11 for humans; OR = 0.05, 0.01-0.17 for animals), pooling method (Peto fixed effects OR = 0.61, 95%CI: 0.34-1.11 for humans; OR = 0.07, 0.03-0.15 for animals), and statistical model (fixed effects M-H RR = 0.72, 95%CI: 0.48-1.08 for humans; RR = 0.24, 0.13-0.44 for animals) showed similar findings.

As shown in Supplemental Figure 1, we observed publication bias and found none in the clinical trials, and little in the preclinical experiment studies. However, it remains unclear because of the small number of included studies.

Bias risk of included RCT and non-RCT studies

By bias assessment, all the included RCT studies were regarded as low or middle-risk overall, with 4 to 6 of 7 items listed by the Cochrane assessment standards for RCT assessed as low risk (Supplemental Table 2). For non-RCT studies, all the clinical trials among humans scored 5 or 6 (total score: 9), indicating a low or middle risk, while those of pre-clinical experiments scored a little higher, with most scoring 7 or 8 (Supplemental Table 3).

DISCUSSION

Main findings and interpretations

By conducting a systematic review of 18 clinical trials and 12 preclinical experiment in large animals, including a meta-analysis of selected studies, we suggest that the BAL might reduce mortality from ALF in large animals, but not in humans [BAL *vs* control: RR (95%CI), 0.27 (0.12-0.62) for animals and 0.72 (0.48-1.08) for humans], with no heterogeneity observed between included studies. Compared with the preclinical experiments, most of the clinical trials were conducted more than 10 years ago. Moreover, the BAL used in large animals has undergone an obvious improvement regarding the type, cell source, cell mass, and bioreactor. All the studies showed improvements in liver and renal functions, hematologic and coagulative parameters,

Table 2 Outcomes and adverse events after treating by bioartificial liver support system in clinical trials

ID	Treating time	Bridging time	OLT /total	SR of OLT	SR of no OLT	Recovery without OLT	Follow-up time	Hemodynamics	Liver and renal function	Hematologic and coagulation	Encephalopathy index	Neurologic	Adverse events	PERV test
1	1.6 × 6 h	24 h	7/7	100%	NA	NA	NR	Stable	Decreased: Ammonia; transaminases	Decreased: Fibrinogen	Improved	Decreased: ICP Increased: CPP	None	NR
2	2.1 × 6 h	45.3 h	16/18	100%	50%	1	NR	Stable	Decreased: Ammonia; ALB; transaminases; bilirubin; BUN; creatinine Increased: Glucose	Not improved	Improved	Decreased: ICP; Increased: CPP; GCS; CLOCS	Transient hypotension	NR
	1.6 × 6 h	83.0 h	3/3	100%	NA	NA	NR	Stable	Decreased: Transaminases; bilirubin; ALB Increased: Glucose	Not improved	Not improved	Not improved		
3	1.8 × 6 h	38.6 h	14/15	100%	100%	1	2 m-62 m	Stable	Decreased: Ammonia; bilirubin; transaminases; ALB; BUN; creatinine; Increased: Glucose	Not improved	Improved	Decreased: ICP; Increased: CLOCS	None	Negative 5 yr
	1.7 × 6 h	21 h-8 d	3/3	100%	NA	NA	2 m-62 m	Stable	Not improved	Not improved	Improved	Not improved		
4	1.9 × 6 h	46 h	10/10	80%	NA	NA	24.5 m	Stable	Decreased: Bilirubin; transaminases	Decreased: Platelets; Fibrinogen	Improved	Increased: GCS; CLOCS	Bleeding; Transitory hypotension	Negative
5	52.6 h	40.8 h	4/11	100%	14%	1	10 d	Stable	Improved	NR	Improved	Improved	Transitory hypotension	NA
6	50.8 h	50.8 h	5/5	80%	NA	NA	30 d	Stable	Improved	NR	Not improved	Not improved	None	NA
7	18.9 h	NR	6/7	100%	100%	1	18 m	Stable	Decreased: Bilirubin; Ammonia	NR	NR	Improved	Transitory hypotension	Negative 18 m
8	35 h	35 h	1/1	100%	NA	NA	15 m	Stable	Decreased: Bilirubin; Ammonia; Lactate	Not improved	NR	Improved	NR	Negative 12 m
9	1.7 × 6 h	39.3 h	12/12	100%	NR	NR	NR	Stable	Decreased: Bilirubin; Ammonia; Transaminases; Increased: Glucose	Not improved	Improved	Decreased: ICP Increased: CPP; CLOCS	None	NR
10	27.3 h	NR	8/8	100%	NR	NR	3 yr	Stable	Decreased: Bilirubin; ALB	Decreased: Platelets	Improved	NA	Decreased body temperature	Negative 3 yr
11	79 h	79 h	1/1	100%	NR	NR	1 yr	Stable	Decreased: Lactate; BUN; creatinine	Improved	Improved	Improved	Decreased body temperature	NA
12	12 h	7 d	NA[1]	NA[1]	NA[1]	NA[1]	7 d	Stable	Decreased: Lactate; Ammonia; Transaminases	Improved	NR	Not improved	Hypoglycemia	NR
13	11.7 h	11.7 h	6/7	83%	0%	0	NA	Stable	Decreased: Bilirubin; Transaminase; Ammonia; Lactate; Urea	Improved	NR	Improved	None	Negative 180 d
14	1.1 × 6 h	NR	NA[2]	NA[2]	NR	NR	1 m	Stable	Decreased: Bilirubin; Transaminase; Ammonia	Improved	NR	Improved	None	NR
15	2.9 × 6 h	NR	3/8	100%	100%	5	NR	Stable	Decreased: Ammonia; Bilirubin; Transaminases; BUN; Creatinine	Improved	Improved	Decreased: ICP Increased: CLOCS	None	NR
16	NR	NR	2/6	100%	75%	3	NR	NR	Decreased: Ammonia	Not improved	NR	Improved	Coagulopathy pneumonia, sepsis, and diseased progression	Negative
17	72 (3-168) h	NR	2/9 *vs* 2/8[3] 1/3 *vs* 1/4[3]	50% *vs* 50%[3] 100% *vs* 100%[3]	6/7 *vs* 5/6[3] 0% *vs* 0%[3]	6 *vs* 5[3] 0 *vs* 0[3]	NR	Stable	Decreased: Ammonia	Improved	NR	NR	Tachypnoeic; Tachycardic; Pyrexial;Disseminated intravascular coagulation	NA

18	17.4 h	5 d *vs* 3 d[3]	45/85 *vs* 49/86[3]	89% *vs* 80%[3]	50% *vs* 38%[3]	20 *vs* 14[3]	30 d	Stable	Decreased: Bilirubin	Not improved	NR	Not improved	None	Negative 12 m

[1]Suitable liver was available for OLTx during that time;
[2]Simultaneous HBAL 1/7 died; nonsimultaneous HBAL 2/5 died;
[3]Intervention *vs* control. NA: Not applicable; NR: Not reported; SR: Survival rate; OLT: Orthotopic liver transplantation; ALB: Albumin; BUN: Blood urea nitroge; ICP: Intracranial pressure; CPP: Cerebral perfusion pressure; GCS: Glasgow coma score; CLOCS: Comprehensive level of consciousness score; PERV: Porcine endogenous retrovirus.

Table 3 Characteristics of preclinical experiments on large animals in past 10 years

ID	Year	Animal				BAL system					Outcomes		Test for PERV
		Species	No	Sex	Weight (kg)	Inducer	Type	Cell	Mass	Bioreactor	Treatment time	Survival	Other	
19	2011	Pig	30	M	10-15	D-galactosamine	FBBAL	Alginate-chitosan encapsulated primary porcine hepatocytes	5×10^9	Choanoid fluidized bed bioreactor	6 h	Survival time: BAL 72.9 ± 4.72 h Sham BAL 54.6 ± 4.09 h Control 54.8 ± 3.98 h	Decreased: Lactate; Glucose	NR
20	2012	Canine	32	NR	10-15	D-galactosamine	HBAL	Co-cultured porcine hepatocytes and bone marrow mesenchymal stem cells	$1x\ 10^{10}$	Multi-layer flat-plate bioreactor + anionic resin adsorption column	3 h	7 day survival: HBAL 7/8 BAL 5/8 NBAL 4/8 Control 3/8	Decreased: Transaminases; LDH; Ammonia; Bilirubin; PT Increased: ALB	Negative 7 d
21	2013	Pig	13	F	25-30	Hepaticartery ligation	NA	Alginate-encapsulated HepG2cell-spheroids	5×10^{10}	Fluidised-bed bioreactor	7 h	Survival time: BAL 10.5 ± 20.7 h SBAL 8.6 ± 21.4 h	Decreased: ICP; Ammonia; Increased: Bilirubin; Acidosis	NR
22	2014	Cynomolgus monkey	15	M	6.5-7.0	D-galactosamine	HBALSS	Human hepatic CL-1 cells grown in microgravity culture	4×10^9	Perfusion bioreactor	6 h	Survival time: 128 h BAL 5/10 Control 0/5	Decreased: bilirubin; TBA; BUN; Cr; ammonia; Fischer indices Increased: ALB	NR
23	2015	Canine	8	NR	10-13	D-galactosamine	HBAL	Co-cultured porcine hepatocytes and bone marrow mesenchymal cells	1×10^{10}	Multi-layer flat-plate bioreactor + anionic resin adsorption column	6 h	1 year survival: 7/8	Decreased: Transaminases; PT; Bilirubin; LDH; Ammonia; Increased: ALB	Negative 1 yr
24	2015	Pig	18	F	45	D-galactosamine	SRBAL	Porcine hepatocytes spheroids	200 g	Spheroid reservoir	24 h	Survival 90 h (%) ST 0/6, 0% ST + Non-cell 1/6,17% ST + SRBAL 5/6, 83%	Decreased: Ammonia; ICP; Brain water content	NR
25	2016	Pig	21	M	10-15	D-galactosamine	FBBAL	Alginate-chitosan encapsulated porcine hepatocytes	5×10^9	Choanoid fluidized bed bioreactor	6 h	Survival time: FBBAL 70.4 ± 11.5 h Sham FBBAL 51.6 ± 7.9 h Control 49.3 ± 6.6 h	Decreased: PCs; LPCs; FAs; SM; Increased: CBAs	NR

26	2016	Pig	20	NR	15-25	D-galactosamine	HiHep-BAL	HiHeps	3×10^9	Multi-layer radial-flow bioreactor	3 h	Survival 7 d: hiHep-BAL 7/8 Empty-BAL 1/6 No-BAL 0/6	Decreased: Transaminase Ammonia; Bilirubin; PT	NR
27	2017	Pig	25	F	30-35	Surgical ligation of all blood flow to the liver	UCLBAL	3-dimensional (3D) HepG2-cell spheroids	7.3×10^{10}	Fuidised bed bioreactor	6 h	Survival time: Control-BAL 7.04 ± 1.9 h, (n = 15) Cell-BAL 8.21 ± 2.3 h, (n = 13)	Decreased: PT; INR; ICP; Ammonia	NR
28	2017	Pig	15	M	45-55	Complete hepatic inflow devascularization	NA	Ca-alginate-immobilized hepatocyte spheroids	2×10^{10}	Packed-bed bioreactor	12 h	Median survival time: Control 21 h (n = 5) BAL 28.5 h (n = 5) Cell-free BAL 21 h (n = 5)	Decreased: Ammonia; Creatinine ICP; BP Increased: Urine	NR
29	2018	Rhesus monkey	30	M	10-20	α-amanitin and lipopolysaccharide	SRBAL	Pig hepatocyte-HUVEC organoids	2.6×10^{10}	Spheroid reservoir	6 h	Median survival time: SRBAL 12 h 336 h (n = 6) SRBAL 24 h 248 h (n = 6) SRBAL 36 h 132 h (n = 6) Sham no-cell SRBAL 12 h 90 h (n = 6) Control 60.5 h (n = 6)	Decreased: Ammonia; Bilirubin Increased: Albumin	Negative 6 h
30	2019	Pig	18	F	20-30	85% hepatectomy	SRBAL	Porcine hepatocyte spheroids	200 g	Spheroid reservoir	24 h	Survival rate at 90 h: SMT 0/6 SMT plus no-cell SRBAL 0/6 SMT plus SRBAL (200 g) 5/6	Decreased: Ammonia; ICP; INR Increased: Volume regeneration	NR

NA: Not applicable; NR: Not reported; BAL: Bioartificial liver; Sex: Male (M) and Female (F); ELAD: Extracorporeal liver assist device; AMC-BAL: Academic medical center bioartificial liver; MELS: Modular extracorporeal liver support; BLSS: Novel bioartificial liver support system; RFB-BAL: Radial-flow bioreactor bioartificial liver; HBAL: Hybrid bioartificial liver; FBBAL: Fluidized bed BAL; HBALSS: Hybrid bioartificial liver support system; SRBAL: Spheroid reservoir bioartificial liver; hiHep: Human-induced hepatocytes; CBAs: Conjugated bile acids; PCs: Phosphatidylcholines; LPCs: Lysophosphatidylcholines; FAs: Fatty acids; SM: Sphingomyelin; ICP: Intracranial pressure; ALB: Albumin; PT: Prothrombin time; INR: International normalized ratio; PERV: Porcine endogenous retrovirus.

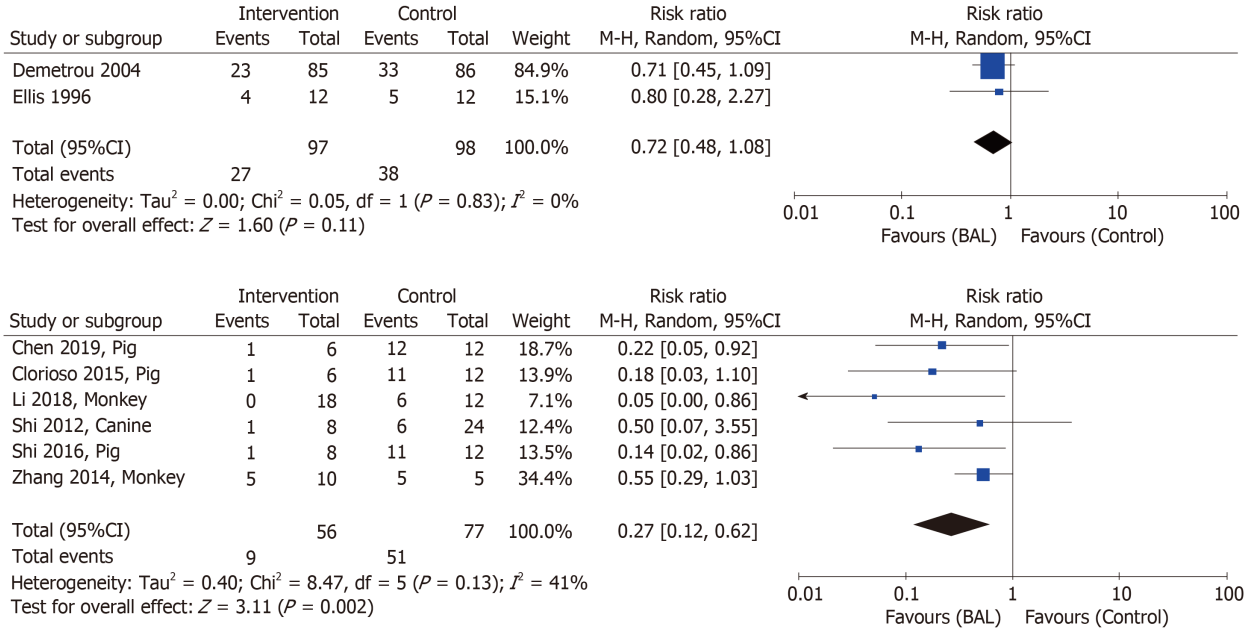

Figure 2 Effect of bioartificial liver support systems on mortality in acute liver failure humans and large animals. The randomized controlled trials for meta-analysis included 2 clinical trials and 6 preclinical experiments with death events (or survival rate) recorded between 90 h to 7d after application of bioartificial liver support systems. M-H: Mantel-Hanszel; BAL: Bioartificial Liver; CI: Confidence interval.

encephalopathy index, and neurological indicators after the treatment with BAL, with neither significant adverse events nor PERV infection.

At present, whether the BAL is able to reduce mortality in the ALF population remains controversial. For example, a meta-analysis performed in 2011[8] indicated that BAL appeared to affect mortality in patients with ALF, while another three meta-analyses of clinical controlled trials conducted by Liu *et al*[37], Kjaergard *et al*[3], and Zheng *et al*[38] demonstrated that the use of the BAL was not associated with the improvement of survival outcome among patients with ALF, which is consistent with our study.

The quality of the BAL is the most important indicator that affects the outcome and adverse events of BAL treatment for ALF, which might support the effect difference between clinical trials and preclinical experiments. As reported in our study, cell source, culture mode, cell mass, and the bioreactor of the BAL were different between the two types of study.

There are currently four main sources of cells for the BAL and their pros and cons are as follows: (1) Human primary hepatocytes are the most suitable cells but are limited by low availability due to a shortage of donor organs[38]; (2) Immortalized human hepatoblastoma cell lines (HepG2/C3A/hepatic CL-1) are sufficiently expanded but are considered to have less metabolic functions than primary hepatocytes[39]; (3) Human-induced hepatocytes (hiHep) were reported to have a potential for metabolic detoxification[12], but it is difficult to meet the demands on a clinical scale because of the cost and complexity of hiHep; and (4) Porcine hepatocytes are the main cells used in the BAL and have similar function with human hepatocytes, are readily available, and are low-cost. Although no PERV infection has been found in 42 patients with long-term immunosuppression and 13 healthcare workers after a follow-up of 5-8 years by a new highly sensitive and specific quantitative real-time polymerase chain reaction assay[40,41], xenozoonosis and the potential risks of PERV infection after treatment remain a concern.

In addition, primary hepatocytes easily lose their function *in vitro* during long-term monolayer culture[42]. In the preclinical experiments, to maintain and improve the viability and metabolic functions of hepatocytes, cells were cultured in a three-dimensional environment to simulate microgravity to form spheroids[30,32,36] or organoids[10], and alginate-chitosan encapsulated spheroids[5,11,33]. Hepatocytes were also co-cultured with bone marrow mesenchymal cells and human umbilical vein endothelial cells to maintain the function of porcine hepatocytes by providing cell-to-cell interactions[10,29,31,43]. In addition, to adapt to the changes of the cells, bioreactors were modified as spheroid reservoirs and multi-layer radial-flow bioreactors to provide a suitable environment for hepatocytes to survive and maintain their cell functions.

Adequate liver cell mass is another crucial indicator for evaluating the BAL, and the innovation requires a higher number of hepatocytes and enhanced function during long-term culture[44]. It has been widely suggested that approximately 30% of the total liver volume is required for survival and that 10-40 billion liver cells without loss of function would be required for BAL treatment[45,46]. Therefore, the low functionality and availability of cells for the clinical scale mass of all the BAL might explain the insignificant effect based on the meta-analysis of the two controlled clinical trials in comparison with the preclinical experiments.

Furthermore, in the preclinical experiments, the subjects in each study were the homogeneous ALF models, but in the clinical trials, the etiologies of ALF varied and were complex, which might have led to different effects of the BAL. In comparison to the patients with PNF, the patients with FHF showed a strikingly different effect of the BAL treatment in a randomized multicenter controlled trial[6] and two non-controlled clinical trials[14,15]. Only unremarkable metabolic effects were observed in the patients with PNF, without amelioration of the neurological state and survival benefit after BAL treatment, whereas an improvement in neurological state and benefit were observed in the patients with FHF, even though one case report on PNF showed a great improvement in neurological state with a change in coma stage from IV to I after treatment with MELS[22].

Strengths and limitations

Our study has several strengths. First, by using a systematic searching strategy and selection procedures, we included all the clinical trials of BAL for ALF and preclinical experiments on large animals in the recent decade, which might represent a current overview of research in this domain, making our study probably the first review to provide evidence for future research. Second, we calculated the combined effect of BAL for ALF by performing a meta-analysis of RCT studies stratified according to clinical trial and preclinical experiment, making the effects comparable between the two study types; meanwhile, the bias of all the included studies was assessed as low to middle-risk and the publication bias was subtle. Finally, we created a detailed checklist of all the potential information associated with the outcome of BAL. Two independent reviewers conducted data extraction, ensuring quality data and allowing for examination of the gap between preclinical experiments and clinical trials.

Our study has two main limitations. One is that the number of RCT clinical trials included was limited to meta-analyses even if many studies have been conducted on ALF and other liver diseases such as acute-on-chronic liver failure. Nevertheless, our data could be usable. Thus, the overall effect of BAL for ALF in humans was desirable but must be verified in the future. Another limitation is that it was not necessary or proper for us to use a meta-regression for controlling covariates, because there existed no heterogeneity between included studies and the limited number of included studies did not meet the requirement of the precondition for regression. Thus, we could not provide further evidence for future research and practice.

Suggestions for future research

Based on the results of our study, we suggest the following for future clinical trials, preclinical experiments, and transformations. First, alternative cells or methods for acquiring high-quality liver cells *in vitro* must be identified to achieve clinical-scale goals. Second, the effects of the subgroups, patients with PNF or FHF, and patients with different etiologies should be determined and examined in clinical trials in the future. Finally, the advanced BAL, which proved to have a significant benefit on the survival outcome of the large-animal ALF model, should undergo clinical transformation as much as possible.

ARTICLE HIGHLIGHTS

Research background

Acute liver failure (ALF) has a high mortality varying from 80% to 85% with rapid progress in multi-organ system failure. Bioartificial liver (BAL) support systems have a potential effect to provide temporary support to bridge patients with acute liver failure to liver transplantation or spontaneous recovery. In the past decades, several BAL support systems have been conducted in clinical trials, but remained verified. More recently, concerns have been raised on the renovation of high-quality cell sources and configuration of BAL support systems to provide more benefits to ALF models in preclinical experiments.

Research motivation

A systematic review and meta-analysis of the existing literature on the use of BAL among humans and large animals with ALF could help bridge the gap between preclinical experiments and clinical trials regarding the effect of BAL for treating acute liver failure.

Research objectives
To investigate the characteristics of studies about BAL for ALF, and to evaluate their effects on mortality.

Research methods
Eligible clinical trials and preclinical experiments on large animals were identified on Cochrane Library, PubMed, and EMbase up to March 6, 2019. Two reviewers independently extracted the necessary information, including the key BAL indicators, survival and indicating outcomes, and adverse events during treatment. Descriptive analysis was used to identify the characteristics of the included studies, and a meta-analysis by including only RCT studies was performed to combine the overall effect of BAL on mortality among humans and large animal, respectively.

Research results
Of 30 selected studies, 18 were clinical trials and 12 were preclinical experiments. The meta-analysis results suggested that BAL might reduce the mortality of ALF in large animals, probably due to the recent improvement of BAL, including the type, cell source, cell mass, and bioreactor, but seemed ineffective for humans. Liver and renal functions, hematologic and coagulative parameters, encephalopathy index, and neurological indicators seemed to improve after BAL, with neither meaningful adverse events nor porcine endogenous retrovirus infection.

Research conclusions
BAL may reduce the mortality of ALF by bridging the gap between preclinical experiments and clinical trials. Clinical trials using improved BAL must be designed scientifically and conducted in the future to provide evidence for transformation.

Research perspectives
Our study could provide some suggestions for future clinical trials, preclinical experiments, and transformations. First, alternative cells or methods for acquiring high-quality liver cells *in vitro* must be identified to achieve clinical-scale goals. Second, the effects of the subgroups, patients with PNF or FHF, and patients with different etiologies should be determined and examined in clinical trials in the future. Finally, the advanced BAL, which proved to have a significant benefit on the survival outcome of the large-animal ALF model, should undergo clinical transformation as much as possible.

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META-ANALYSIS

Immunotherapy with dendritic cells and cytokine-induced killer cells for hepatocellular carcinoma: A meta-analysis

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Abstract

BACKGROUND

Hepatocellular carcinoma (HCC) has been revealed as the second most common cause of cancer-related deaths worldwide. The introduction of cell-based immunotherapy, including dendritic cells (DCs) and cytokine-induced killer cells (CIKs), has brought HCC patients an effective benefit. However, the efficacy and necessity of cellular immunotherapy after different interventional therapy remains to be further explored.

AIM

To investigate the efficacy of cellular immunotherapy, involving DCs and CIKs, combined with different conventional treatments of HCC.

METHODS

We performed a literature search on PubMed and Web of Science up to February 15, 2019. Long-term efficacy (overall survival and recurrence) and short-term adverse effects were investigated to assess the effectiveness of immunotherapy with DCs and/or CIKs. Review Manager 5.3 was used to perform the analysis.

RESULTS

A total of 22 studies involving 3756 patients selected by eligibility inclusion criteria were forwarded for meta-analysis. Combined with the conventional clinical treatment, immunotherapy with DCs and/or CIKs was demonstrated to significantly improve overall survival at 6 mo [risk ratio (RR) = 1.07; 95% confidence interval (CI): 1.01-1.13, $P = 0.02$], 1 year (RR = 1.12; 95%CI: 1.07-1.17, $P < 0.00001$), 3 years (RR = 1.23; 95%CI: 1.15-1.31, $P < 0.00001$) and 5 years (RR = 1.26; 95%CI: 1.15-1.37, $P < 0.00001$). Recurrence rate was significantly reduced by cellular immunotherapy at 6 mo (RR = 0.50; 95%CI: 0.36-0.69, $P < 0.0001$) and 1 year (RR = 0.82; 95%CI: 0.75-0.89, $P < 0.00001$). Adverse effect assessment addressed that immunotherapy with DCs and/or CIKs was accepted as a safe, feasible treatment.

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CONCLUSION
Combination immunotherapy with DCs, CIKs and DC/CIK with various routine treatments for HCC was evidently suggested to improve patients' prognosis by increasing overall survival and reducing cancer recurrence.

Key words: Hepatocellular carcinoma; Immunotherapy; Dendritic cells; Cytokine-induced killer cells

Core tip: Hepatocellular carcinoma has been revealed as the second most common cause of cancer-related deaths worldwide. Though several analyses have supported the effective benefit of cellular immunotherapy when combined with specific hepatocellular carcinoma treatment, the efficacy and necessity of cellular immunotherapy after different interventional therapy remains to be interrogated. Our study suggested that the combination of immunotherapy with dendritic cells, cytokine-induced killer cells and a combination of the two with various routine hepatocellular carcinoma treatments could evidently improve patients' prognosis by increasing the overall survival and reduce the recurrence of the malignancy.

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INTRODUCTION

Liver cancer, approximately 75% of which is ascribed to hepatocellular carcinoma (HCC), has been revealed as the second most common cause of cancer-related deaths worldwide with around 745000 deaths annually[1]. Both the incidence and mortality are rising worldwide. Cirrhosis, hepatitis B virus and hepatitis C virus are commonly regarded as the main risk factors for HCC.

Curative treatment is achieved by surgical resection, orthotopic liver transplantation, transarterial chemoembolization (TACE) and local percutaneous tumor ablation. However, these curative approaches are only accessible to limited numbers of patients, as most are diagnosed at advanced stages with unresectable tumors. Although much progress has been made with promising phase II trial results, drug development has been disappointing with many failures in phase III trials[2]; hence, few drugs have been approved so far by the United States Food and Drug Administra-tion. A multitarget tyrosine kinase inhibitor, sorafenib, the only approved systemic therapy, achieves an increased survival of only up to 3 mo[3,4].

Immunotherapy was introduced into the field as the ability to escape from immunological surveillance, which forms the basis for tumor progression. The underlying mechanism comprises of defective antigen presentation, dysfunction of effector T cells, cytokine disarray and alterations in immune checkpoints[5]. While conventional chemotherapy exerts its effect by directly reducing tumor volume morphologically, immunotherapy works in an indirect way and takes longer to induce an effective immune response. However, it provides a more durable antitumor effect. Immune-based approaches include cytokines, vaccines, adoptive cell therapy [based on peripheral blood mononuclear cells or dendritic cells (DCs)][6-8], tumor-antibody-based immunotherapy[9] and immune checkpoint inhibitors. Recombinant interferon-α was the first immunotherapeutic agent introduced into the field, although, even with its features of immunostimulation and antiangiogenesis, it failed to show a significant effect in clinical trials of patients with HCC[10,11].

Vaccine strategies are carried out on different anti-cancer platforms, including RNA-, peptide- and protein-based vaccines, whole-tumor-cell vaccine, and most widely, DC-based vaccines. DC-based vaccines are adapted more for solid malignancies, including melanoma, renal cancer and prostate cancer, as well as HCC[12,13]. As mature DCs prime T cells and boost memory T cells, induction of DC maturation by Toll-like receptor ligand or cytokines is often applied clinically. Cross-

presentation, the process of DCs presenting captured antigen to $CD8^+$ cells via major histocompatibility complex class I, is regarded as a critical step in the efficient induction of antitumor cytotoxic response[12]. An important subtype of DCs was identified in 2010 on account of its high capability in cross-presentation with high expression of CD141 and Toll-like receptor 3. These $CD141^+$ DCs yielded high amounts of type I interferon under stimulation with polyinsionic-polycytidylic acid, which leads to a vigorous T helper 1 cell response; hence the effective anticancer response[14]. Therefore, polyinsionic-polycytidylic acid and its derivatives are adopted as adjuvants for DC-based vaccines.

Adoptive cell therapy is commonly performed with cytokine-induced killer cells (CIKs), tumor-infiltrating lymphocytes and genetically modified T cells. Among these, CIKs have been used in more clinical trials. CIKs, consisting of $NKG2D^{high}$ T cells, activated natural killer cells and natural killer T cells[15], are generated *ex vivo* from peripheral blood mononuclear cells and stimulated with cytokines and antibodies targeting CD3. CIKs harbor high capacity of proliferation and have a cytolytic effect against cancer cells. Although several analyses have supported the beneficial effect of cellular immunotherapy when combined with specific HCC treatment[16,17], the efficacy and necessity of cellular immunotherapy after different interventional therapy remains to be interrogated. In this study, a systematic review and meta-analysis was performed to investigate the efficacy of cellular immunotherapy, involving DCs, CIKs and DC/CIK combination therapy, combined with different treatments of HCC.

MATERIALS AND METHODS

Systematic literature search

We conducted a widespread literature search on PubMed and Web of Science Core Collection. Articles up to February 15, 2019 were filtered out by key words including hepatocellular OR liver AND cancer OR tumor OR tumour OR carcinoma OR neoplasm AND immunotherapy OR immune checkpoint OR immunotherapeutic. Study selection was conducted by two independent investigators. Discrepancies were resolved by discussion and consensus. Full text was retrieved for further decision if the abstract was insufficient to support the inclusion criteria. Three reviewers independently evaluated studies for eligibility.

Inclusion and exclusion criteria

Randomized controlled trials (RCTs) and controlled trials were taken into consideration, but trials with only safety data, animal studies and *in vitro* studies were excluded. Studies that met the following criteria were included: (1) Clinical trials on immunotherapy for HCC patients with full text and available data in English; and (2) Clinical trials providing survival data [disease-free survival, progression-free survival, and overall survival (OS)] and adverse effects.

Data extraction and quality assessment

The following information was extracted for each article: last name of first author, year of publication, phase of clinical trial, number of enrolled subjects, treatment arms, number of patients in control and conventional groups, OS, recurrence rate and adverse effects. The quality assessment was performed with Cochrane Collaboration's tool for RCT trails and MINORS[18] for non-RCT cohort studies.

Statistical analysis

We conducted all the analysis with Review Manager 5.3 (Cochrane Collaboration). Risk ratio (RR) was calculated to assess the effect of interventions. Results were presented with 95% confidence interval (CI). We performed Cochrane's Q statistic ($\chi 2$) to assess the heterogeneity of the trials and the I^2 statistic for inconsistency. $P < 0.05$ or $I^2 > 50\%$ was considered an invalid assumption of homogeneity. A random-effects model was applied for clinical trials of significant heterogeneity, otherwise a fixed-effects model was applied. Assessment of potential publication bias was evaluated by funnel plot, where two-tailed $P < 0.05$ was considered statistically significant.

RESULTS

Description of included trials

A total of 2643 citations were identified from the primary literature search. Among them, 2621 studies were excluded for the following reasons: overlapping studies, reviews, no access to full-text in English, letters, and individual case studies.

According to the inclusion criteria, 22 studies (Figure 1) including 3756 patients were adopted in the meta-analysis. Five of the included studies involving 390 patients were aligned for analyzing the effect of mono-immunotherapy with DC-based vaccines. A total of 3211 patients involved in 13 trials were assigned for comparative analysis of mono-immunotherapy with CIKs. The other four trials with 155 patients focused on the effect of combined approach with DC vaccines and CIKs (Table 1). Most of those trials evaluated the effect of immunotherapy based on interventions for primary tumor ablation by surgical resection, radiofrequency ablation and cryoablation. Five studies[19,20,24,26,27] were conducted with patients receiving TACE treatment. A few studies assigned patients with chemotherapy[32] or only supportive treatment[21] as the control group.

Efficacy and prognosis assessment

Compared with the patients in the control group, regardless of original treatment arm, patients receiving monotherapy with DC-based vaccine had a higher 1-year OS (RR = 1.16; 95%CI: 1.03-1.30, $P = 0.01$), although there was no significant improvement in OS at 6 mo or 3 years. Monotherapy with CIKs demonstrated significant improvement in OS at 6 mo (RR = 1.09; 95%CI: 1.03-1.16, $P = 0.005$), 1 year (RR = 1.11; 95%CI: 1.06-1.16, $P < 0.00001$), 3 years (RR = 1.23; 95%CI: 1.15-1.31, $P < 0.00001$) and 5 years (RR = 1.25; 95%CI: 1.14-1.36, $P < 0.00001$). Unsurprisingly, combined therapy with DCs and CIKs also improved OS at 1 year (RR = 3.8; 95%CI: 1.29-11.22, $P = 0.02$) and 5 years (RR = 1.45; 95%CI: 0.99-2.12, $P = 0.05$). Taken together, immunotherapy based on DCs and/or CIKs significantly increased OS at 6 mo (RR = 1.07; 95%CI: 1.01-1.13, $P = 0.02$), 1 year (RR = 1.12; 95%CI: 1.07-1.17, $P < 0.00001$), 3 years (RR = 1.23; 95%CI: 1.15-1.31, $P < 0.00001$) and 5 years (RR = 1.26; 95%CI: 1.15-1.37, $P < 0.00001$) (Figure 2).

Besides the improved OS of patients, reduced tumor recurrence rate was achieved in most trials. DC-based vaccination alone addressed a declining shift in 1-year recurrence (RR = 0.64; 95%CI: 0.44-1.93, $P = 0.02$). Immunotherapy with CIKs showed similar effects on recurrence at 6 mo (RR = 0.45; 95%CI: 0.30-0.66, $P < 0.0001$), 1 year (RR = 0.83; 95%CI: 0.76-0.91, $P < 0.0001$) and 1.5 years (RR = 0.39; 95%CI: 0.18-0.85, $P = 0.02$). The data on recurrence with DC/CIK combined therapy were unfortunately not available. The retardation effect of recurrence was observed at 6 mo (RR = 0.50; 95%CI: 0.36-0.69, $P < 0.0001$) and 1 year (RR = 0.82; 95%CI: 0.75-0.89, $P < 0.00001$) (Figure 3).

Adverse effect analysis

None of the studies reported immunotherapy-related hospital mortality. Only 23 patients in four studies[23,29,32,36] were reported with grade III or IV adverse events. Fever was addressed as the most common event after immunotherapy. Other adverse effects included shivering, vomiting, fatigue, abdominal pain and leukopenia. The overall adverse event rate was higher in immunotherapy groups, but symptomatic remissions were declared within 24 h. In brief, immunotherapy was accepted as a safe, feasible treatment, though with an increased incidence of adverse events.

Quality assessment of trials

Seven studies included in this systematic review were RCTs, including two with DC-based vaccine, three with mono-immunotherapy with CIKs and two with combination immunotherapies. Cochrane Collaboration's tool was used to assess the quality of the RCTs. All these RCTs were verified with high quality (Table 2), and 15 non-RCTs were validated as high quality by MINORS (Table 3).

Publication bias

Funnel plots and Egger's regression test were applied to OS and recurrence rate in order to guarantee the potency of this meta-analysis (Figure 4). Symmetrical distribution of individual studies indicated no evident publication bias.

DISCUSSION

Although surgical resection, transplantation, local tumor ablation and TACE are well accepted with proven survival benefit[41], these clinical treatments of HCC have their limited scope of application according to the tumor progression of individual patients. Besides, tumor recurrence can occur within five years even with curative interventions[42]. Unlike some other solid tumors, no effective neoadjuvant or adjuvant therapy has been validated to reduce the recurrence risk so far[41]. The introduction of immunotherapy, however, brought new hope in this regard. In this meta-analysis, we found that immunotherapy could increase OS and decrease recurrence rate in HCC.

Table 1 Clinical information and characteristics of included studies

Author (year)	Country	Control	Immuno-therapy interven-tions	Patients		Child-Pugh score A/B/C		Immune cell regimens	Course
				Control	Trial	Control	Trial		
Nakamoto (2007)[19]	Japan	TACE	TACE + DC	11	10	4/7/0	8/2/0	5×10^6	1
Nakamoto (2011)[20]	Japan	TACE	TACE + DC	22	13	NR	5/8/0	5×10^6	1
El Ansary (2013)[21]	Egypt	Supportive treatment	DC	15	15	0/14/1	0/15/0	20×10^6	1
Sun (2015)[22]	China	Resection + FOLFOX6	Resection + DC	80	80	NR	NR	8.9×10^9	6
Lee (2017)[23]	South Korea	Resection/RFA/PEI	Resection/RFA/PEI + DC	75	69	NR	NR	3×10^7	6
Weng (2007)[24]	China	TACE + RFA	TACE + RFA + CIK	40	45	33/7/0	36/9/0	$1.0\text{-}1.5 \times 10^{10}$	8/9
Dong (2008)[25]	China	Resection	Resection + CIK	43	84	34/9/0	68/16/0	$1.0\text{-}2.0 \times 10^{10}$	3/5
Hao (2010)[26]	China	TACE	TACE + RFA + CIK	74	72	66/8/0	65/7/0	$1.0\text{-}5.0 \times 10^{10}$	4
Pan (2010)[27]	China	TACE + RFA	TACE + RFA + CIK	39	42	NR	NR	1.0×10^{10}	4
Pan (2013)[28]	China	Resection	Resection + CIK	206	204	206/0/0	204/0/0	$1.0\text{-}1.5 \times 10^{10}$	4
Lee (2015)[29]	South Korea	Resection/RFA/PEI	Resection/RFA/PEI + CIK	112	114	112/0/0	114/0/0	NR	16
Pan (2015)[30]	China	Resection	Resection + CIK	520	511	NR	NR	$1.0\text{-}1.5 \times 10^{10}$	4
Chen (2016)[31]	China	Resection	Resection + CIK	118	231	205/12/0	222/9/0	$1.0\text{-}1.5 \times 10^{10}$	4
Li (2016)[32]	China	Oxaliplatin + Capecitabine	CIK	37	37	33(A + B)/4(C)	27(A + B)/10(C)	NR	NR
Chang (2018)[33]	China	Resection	Resection + CIK	145	145	145/0/0	145/0/0	NR	NR
Lee (2018)[34]	South Korea	Resection/RFA/PEI	Resection/RFA/PEI + CIK	112	114	112/0/0	114/0/0	6.4×10^9	16
Cui (2014)[35]	China	RFA	RFA + NK/γδT/CIK	32	30	14/18/0	18/12/0	$1.2\text{-}2.0 \times 10^9$	8
Qian (2016)[36]	China	RFA	RFA + NK/γδT/CIK	31	73	NR	NR	$1.0\text{-}2.0 \times 10^9$	8
Qiu (2011)[37]	China	Resection	Resection + DC/CIK	9	9	NR	NR	$2.0\text{-}20.0 \times 10^9$	NR
Niu (2013)[38]	China	Cryoablation	Cytotherapy + DC/CIK	12	21	NR	NR	$6.0\text{-}10.0 \times 10^9$	NR
Shimizu (2014)[39]	Japan	Resection	Resection + DC/CIK	40	35	44/8/0	34/8/0	NR	NR
Yu (2015)[40]	China	Microwave ablation	Microwave ablation + DC/CIK	15	14	15/0/0	13/1/0	NR	NR

TACE: Transcatheter arterial chemoembolization; RFA: Radiofrequency ablation; PEI: Percutaneous ethanol injection; DC: Dendritic cell; CIK: Cytokine-induced killer cell; NK: Nature killer cell; NR: Not reported.

We comprehensively analyzed 22 individual studies with 3756 HCC patients in this meta-analysis and illustrated a positive prognostic efficacy of immunotherapy with DC-based vaccine and/or CIK-based adoptive therapy. Our results demonstrated that an extended OS (6 mo, 1, 3 and 5 years) was achieved with the aforesaid immunotherapy based on different HCC interventional therapies. Similar benefits in OS were described in another meta-analysis[43] of adjuvant adoptive immunotherapy, including CIKs, lymphokine-activated killer cells and lymphocytes in HCC patients after surgery. In that study, a significant reduction in mortality and recurrence was observed at 1, 2 and 3 years but not 5 years[43]. However, compared to the slight

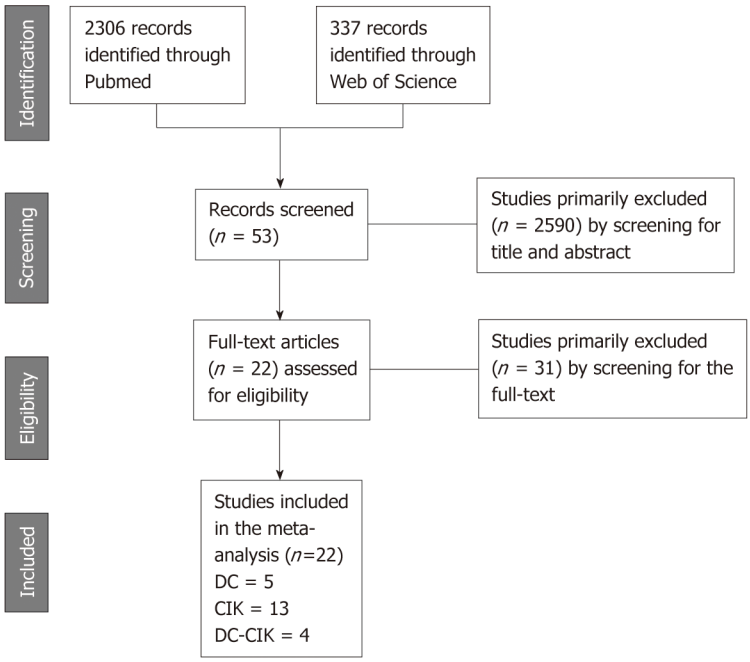


Figure 1 Flow diagram of the literature screening process. DC: Dendritic cell; CIK: Cytokine-induced killer cell.

increase in OS in our study, another systematic review demonstrated a more dramatic shift in OS in TACE-treated HCC patients[16]. The smaller increase in OS in our analysis could have been ascribed to the heterogeneity of the patients included in the controlled trials. Yet, a significant benefit in OS was confirmed regardless of subgroup composition, and more intriguingly, our analysis indicated that short-term recurrence was intensely reduced with immunotherapy independent of the heterogeneity. The clinical benefit of the combined immunotherapy with DCs and CIKs has been demonstrated in many clinical studies[17,44]. This combined approach was reported to have greater antitumor activity *in vitro* than CIK treatment alone[45], while our analysis failed to find sufficient clinical data to support it.

One RCT[22] and one non-RCT cohort study[32] included in this meta-analysis were based on conventional chemotherapy as the control group. Cellular immunotherapy also resulted in improved outcomes, implying a critical role for immunotherapy in patients who have lost their chance for tumor ablation. The combination of chemotherapy and immunotherapy has accumulated positive efficacy in a number of unresectable malignancies[46-48].

Recently, the introduction of immune checkpoint inhibitors has led to a clinical breakthrough in cancer treatment[49]. Six immune checkpoint inhibitors for HCC treatment have been approved by the Food and Drug Administration, and even one (nivolumab) has been introduced as a second-line treatment[50,51] due to its promising effect on immune checkpoints inhibitors in HCC patients. Although some clinical data have presented a good safety profile, disease control and time to progression[50-52], clinical data concerning comparative trials with immune checkpoints in HCC patients are still insufficient for analysis.

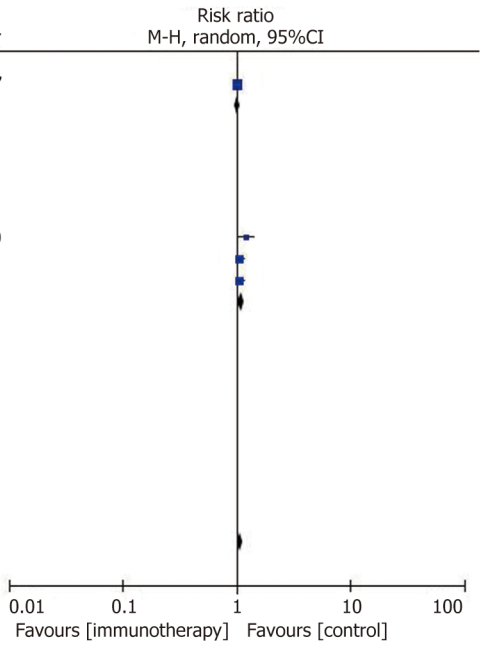
A bias could have been raised due to the low number of available trials and the low number of included patients in the DC-based vaccine group and DC/CIK combination group, which comprised five trials with 390 patients and four trials with 155 patients, respectively. The quality of this meta-analysis was still assured as we have shown no evidence of publication bias.

There were a few limitations to this systematic review and meta-analysis. First, although we included 22 trials in the analysis, the DC and DC/CIK groups comprised only five and four trials, respectively. Most of the trials were conducted in East Asian countries (China, Japan and South Korea); hence, they had less-sufficient statistical power due to the lack of multinational or multiracial clinical data. Second, heterogeneity was observed between the included studies. Factors, including stage of malignancy, different surgical method, number of immunotherapy fusion cycles, and duration of immunotherapy in different clinical centers could have contributed to the heterogeneity.

In conclusion, we demonstrated that the application of immunotherapy with DCs, CIKs and DCs/CIK in addition to various routine HCC treatments could evidently

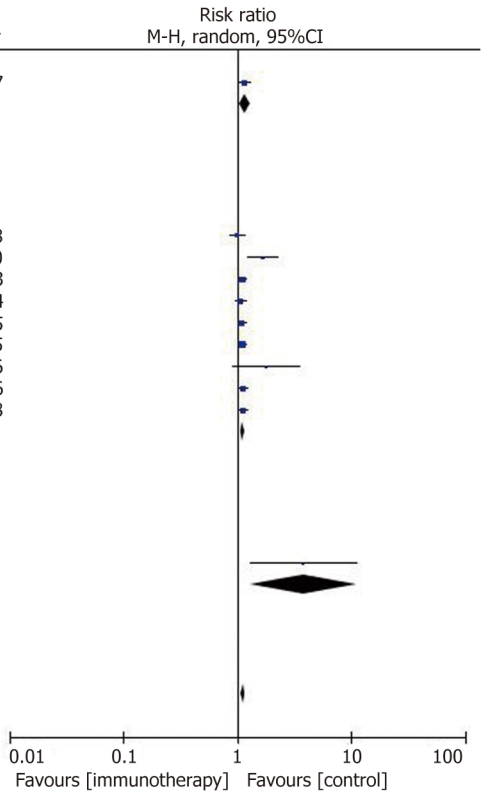
A

Study or subgroup	Immunotherapy Events	Total	Control Events	Total	Weight	Risk ratio M-H, random, 95%CI	Year
2.1.1 DC							
Lee 2017	68	69	72	75	34.6%	1.03 [0.97, 1.08]	2017
Subtotal (95%CI)		69		75	34.6%	1.03 [0.97, 1.08]	
Total events	68		72				
Heterogeneity. Not applicable							
Test for overall effect: Z = 0.95 (P = 0.34)							
2.1.2 CIK							
Hao 2010	65	72	55	74	11.1%	1.21 [1.04, 1.42]	2010
Lee 2015	109	114	100	112	27.1%	1.07 [0.99, 1.15]	2015
Lee 2018	109	114	100	112	27.1%	1.07 [0.99, 1.15]	2018
Subtotal (95%CI)		300		298	65.4%	1.09 [1.03, 1.16]	
Total events	283		255				
Heterogeneity. Tau^2 = 0.00; Chi^2 = 2.62, df = 2 (P = 0.27); I^2 = 24%							
Test for overall effect: Z = 2.80 (P = 0.005)							
2.1.3 DC - CIK							
Subtotal (95%CI)		0		0		Not estimable	
Total events	0		0				
Heterogeneity. Not applicable							
Test for overall effect: Not applicable							
Total (95%CI)		369		373	100.0%	1.07 [1.01, 1.13]	
Total events	351		327				
Heterogeneity. Tau^2 = 0.00; Chi^2 = 6.35, df = 3 (P = 0.10); I^2 = 53%							
Test for overall effect: Z = 2.28 (P = 0.02)							
Test for subgroup differences: Chi^2 = 2.07, df = 1 (P = 0.15); I^2 = 51.8%							



B

Study or subgroup	Immunotherapy Events	Total	Control Events	Total	Weight	Risk ratio M-H, random, 95%CI	Year
2.2.1 DC							
Lee 2017	66	69	62	75	9.8%	1.16 [1.03, 1.30]	2017
Subtotal (95%CI)		69		75	9.8%	1.16 [1.03, 1.30]	
Total events	66		62				
Heterogeneity. Not applicable							
Test for overall effect: Z = 2.48 (P = 0.01)							
2.2.2 CIK							
Dong 2008	55	63	30	34	6.7%	0.99 [0.85, 1.15]	2008
Hao 2010	52	72	32	74	2.3%	1.67 [1.24, 2.25]	2010
Pan 2013	191	204	173	206	15.4%	1.11 [1.04, 1.20]	2013
Cui 2014	30	30	30	32	10.6%	1.06 [0.96, 1.19]	2014
Lee 2015	109	114	97	112	13.5%	1.10 [1.02, 1.20]	2015
Pan 2015	421	511	387	520	16.1%	1.11 [1.04, 1.18]	2015
Li 2016	16	37	9	37	0.5%	1.78 [0.90, 3.50]	2016
Chen 2016	185	231	155	217	10.8%	1.12 [1.01, 1.25]	2016
Lee 2018	109	112	97	112	14.0%	1.12 [1.04, 1.22]	2018
Subtotal (95%CI)		1374		1344	90.0%	1.11 [1.06, 1.16]	
Total events	1168		1010				
Heterogeneity. Tau^2 = 0.00; Chi^2 = 13.17, df = 8 (P = 0.11); I^2 = 39%							
Test for overall effect: Z = 4.61 (P < 0.00001)							
2.2.3 DC - CIK							
Qiu 2011	9	9	2	9	0.2%	3.80 [1.29, 11.22]	2011
Subtotal (95%CI)		9		9	0.2%	3.80 [1.29, 11.22]	
Total events	9		2				
Heterogeneity. Not applicable							
Test for overall effect: Z = 2.42 (P = 0.02)							
Total (95%CI)		1452		1428	100.0%	1.12 [1.07, 1.17]	
Total events	1243		1074				
Heterogeneity. Tau^2 = 0.00; Chi^2 = 19.11, df = 10 (P = 0.04); I^2 = 48%							
Test for overall effect: Z = 4.61 (P < 0.00001)							
Test for subgroup differences: Chi^2 = 5.32, df = 2 (P = 0.07); I^2 = 62.4%							



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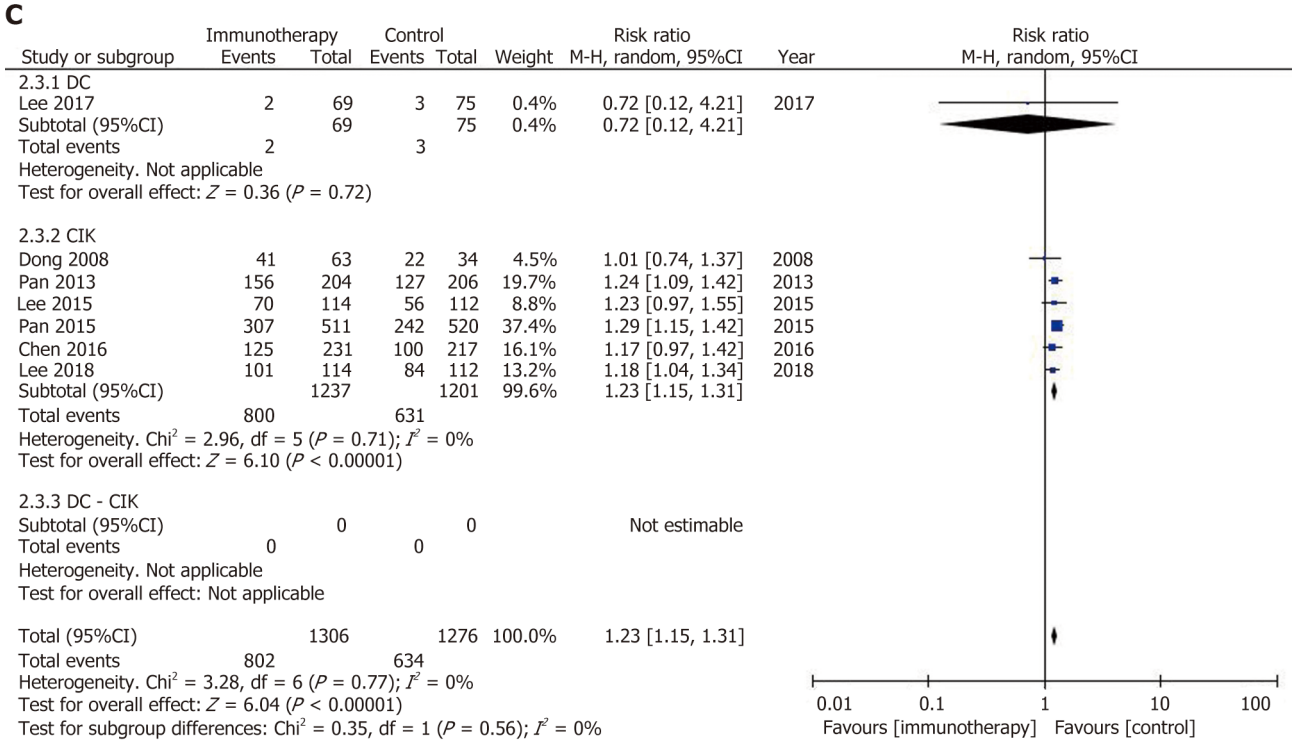

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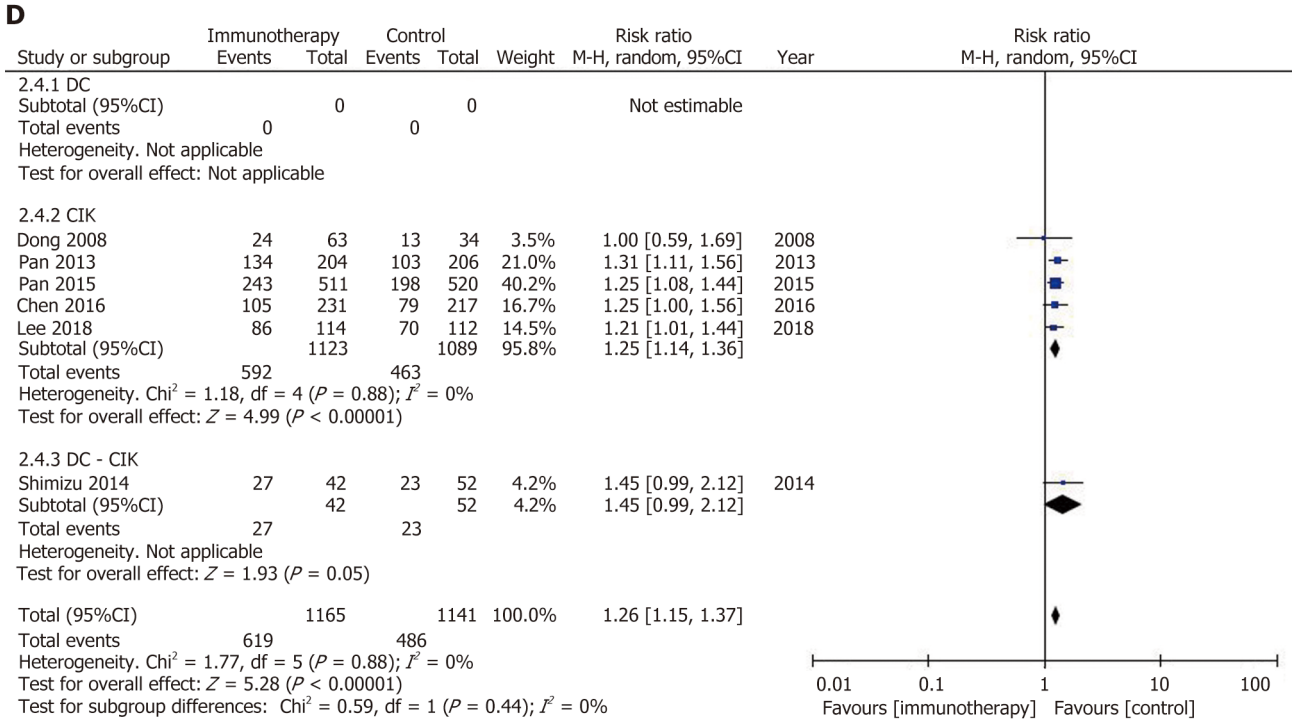


Figure 2 Comparison of 6-mo (A), 1- (B), 3- (C) and 5-yr (D) overall survival between immunotherapy groups and control groups. M-H: Mantel-Haenszel estimates; CI: Confidence interval.

improve patients' prognosis by increasing OS and reducing recurrence. The efficacy of novel immune checkpoint inhibitors based on clinical trials should be assessed in further studies when sufficient data are available. Due to the diverse mechanism behind the immune evasion of tumor cells, approaches combining different immunotherapies might lead to an appealing strategy to treat HCC.

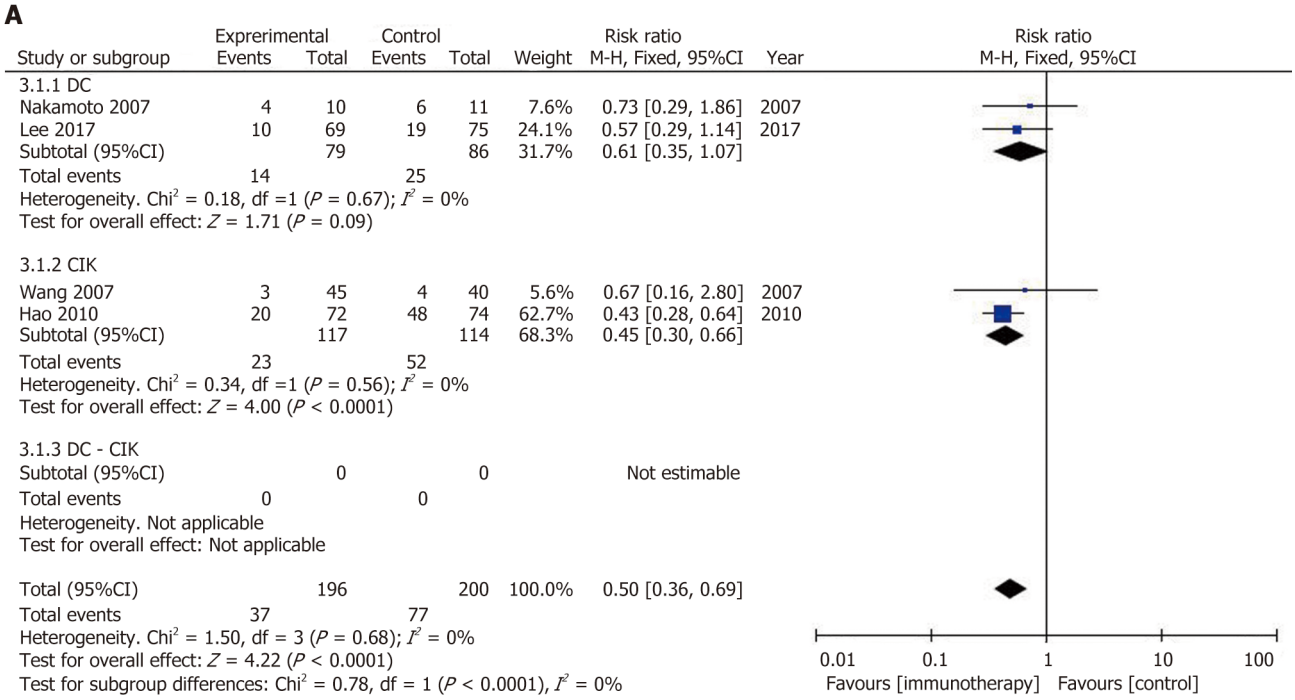
Table 2 Quality assessment of randomized controlled trials with Cochrane Collaboration's tool

Author (year)	Selection	Performance	Detection	Attrition	Reporting	Other
	Bias	Bias	Bias	Bias	Bias	Bias
El Ansary (2013)	Unclear	High	Unclear	Low	Low	Low
Sun (2015)	Unclear	High	Unclear	Low	Low	Low
Dong (2008)	Unclear	High	Unclear	Low	Low	Low
Lee (2015)	Unclear	High	Unclear	Low	Low	Low
Lee (2018)	Unclear	High	Unclear	Low	Low	Low
Qiu (2011)	Unclear	High	Unclear	Low	Low	Low
Yu (2015)	Unclear	High	Unclear	Low	Low	Low

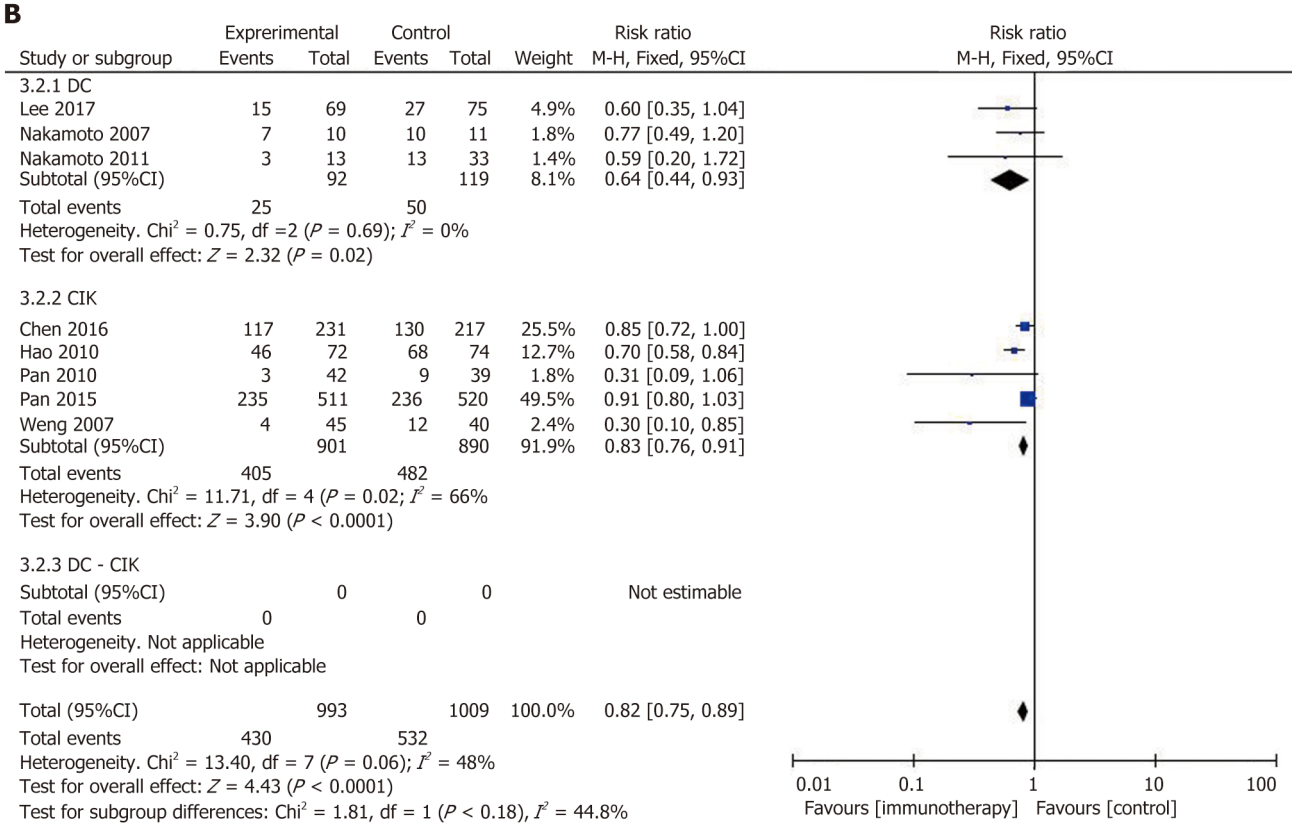
Table 3 Quality assessment of non-randomized controlled trials with MINORS

Author (year)	1	2	3	4	5	6	7	8	9	10	11	12
Nakamoto (2007)	2	2	2	0	0	1	2	0	2	2	2	2
Nakamoto (2011)	2	2	2	2	0	1	2	0	2	2	2	2
Lee (2017)	2	2	2	2	0	2	0	2	2	2	2	2
Weng (2007)	2	2	2	2	0	2	2	0	2	2	2	2
Hao (2010)	2	2	2	2	0	2	2	2	2	2	2	2
Pan (2010)	2	2	2	2	0	1	2	0	2	2	2	2
Pan (2013)	2	2	2	2	0	1	2	0	2	2	2	2
Pan (2015)	2	2	2	2	0	2	2	0	2	2	2	2
Chen (2016)	2	2	2	2	0	2	2	0	2	2	2	2
Li (2016)	2	2	2	2	0	1	2	2	2	2	2	2
Chang (2018)	2	2	2	2	0	2	2	2	2	2	2	2
Cui (2014)	2	2	2	0	0	1	2	0	2	2	2	2
Qian (2016)	2	2	2	1	0	2	0	0	2	2	2	2
Niu (2013)	2	2	2	0	0	2	2	0	2	2	2	2
Shimizu (2014)	2	2	2	0	0	2	2	2	2	2	2	2

A


Exprerimental
Control
Risk ratio
Risk ratio
Study or subgroup Events Total Events Total Weight M-H, Fixed, 95%CI Year
M-H, Fixed, 95%CI
3.1.1 DC
Nakamoto 2007 4 10 6 11 7.6% 0.73 [0.29, 1.86] 2007
Lee 2017 10 69 19 75 24.1% 0.57 [0.29, 1.14] 2017
Subtotal (95%CI) 79 86 31.7% 0.61 [0.35, 1.07]
Total events 14 25
Heterogeneity. Chi² = 0.18, df =1 (P = 0.67); I² = 0%
Test for overall effect: Z = 1.71 (P = 0.09)
3.1.2 CIK
Wang 2007 3 45 4 40 5.6% 0.67 [0.16, 2.80] 2007
Hao 2010 20 72 48 74 62.7% 0.43 [0.28, 0.64] 2010
Subtotal (95%CI) 117 114 68.3% 0.45 [0.30, 0.66]
Total events 23 52
Heterogeneity. Chi² = 0.34, df =1 (P = 0.56); I² = 0%
Test for overall effect: Z = 4.00 (P < 0.0001)
3.1.3 DC - CIK
Subtotal (95%CI) 0 0 Not estimable
Total events 0 0
Heterogeneity. Not applicable
Test for overall effect: Not applicable
Total (95%CI) 196 200 100.0% 0.50 [0.36, 0.69]
Total events 37 77
Heterogeneity. Chi² = 1.50, df = 3 (P = 0.68); I² = 0%
Test for overall effect: Z = 4.22 (P < 0.0001)
Test for subgroup differences: Chi² = 0.78, df = 1 (P < 0.0001), I² = 0%
0.01 0.1 1 10 100
Favours [immunotherapy] Favours [control]

B


Exprerimental
Control
Risk ratio
Risk ratio
Study or subgroup Events Total Events Total Weight M-H, Fixed, 95%CI
M-H, Fixed, 95%CI
3.2.1 DC
Lee 2017 15 69 27 75 4.9% 0.60 [0.35, 1.04]
Nakamoto 2007 7 10 10 11 1.8% 0.77 [0.49, 1.20]
Nakamoto 2011 3 13 13 33 1.4% 0.59 [0.20, 1.72]
Subtotal (95%CI) 92 119 8.1% 0.64 [0.44, 0.93]
Total events 25 50
Heterogeneity. Chi² = 0.75, df =2 (P = 0.69); I² = 0%
Test for overall effect: Z = 2.32 (P = 0.02)
3.2.2 CIK
Chen 2016 117 231 130 217 25.5% 0.85 [0.72, 1.00]
Hao 2010 46 72 68 74 12.7% 0.70 [0.58, 0.84]
Pan 2010 3 42 9 39 1.8% 0.31 [0.09, 1.06]
Pan 2015 235 511 236 520 49.5% 0.91 [0.80, 1.03]
Weng 2007 4 45 12 40 2.4% 0.30 [0.10, 0.85]
Subtotal (95%CI) 901 890 91.9% 0.83 [0.76, 0.91]
Total events 405 482
Heterogeneity. Chi² = 11.71, df = 4 (P = 0.02; I² = 66%
Test for overall effect: Z = 3.90 (P < 0.0001)
3.2.3 DC - CIK
Subtotal (95%CI) 0 0 Not estimable
Total events 0 0
Heterogeneity. Not applicable
Test for overall effect: Not applicable
Total (95%CI) 993 1009 100.0% 0.82 [0.75, 0.89]
Total events 430 532
Heterogeneity. Chi² = 13.40, df = 7 (P = 0.06); I² = 48%
Test for overall effect: Z = 4.43 (P < 0.0001)
Test for subgroup differences: Chi² = 1.81, df = 1 (P < 0.18), I² = 44.8%
0.01 0.1 1 10 100
Favours [immunotherapy] Favours [control]

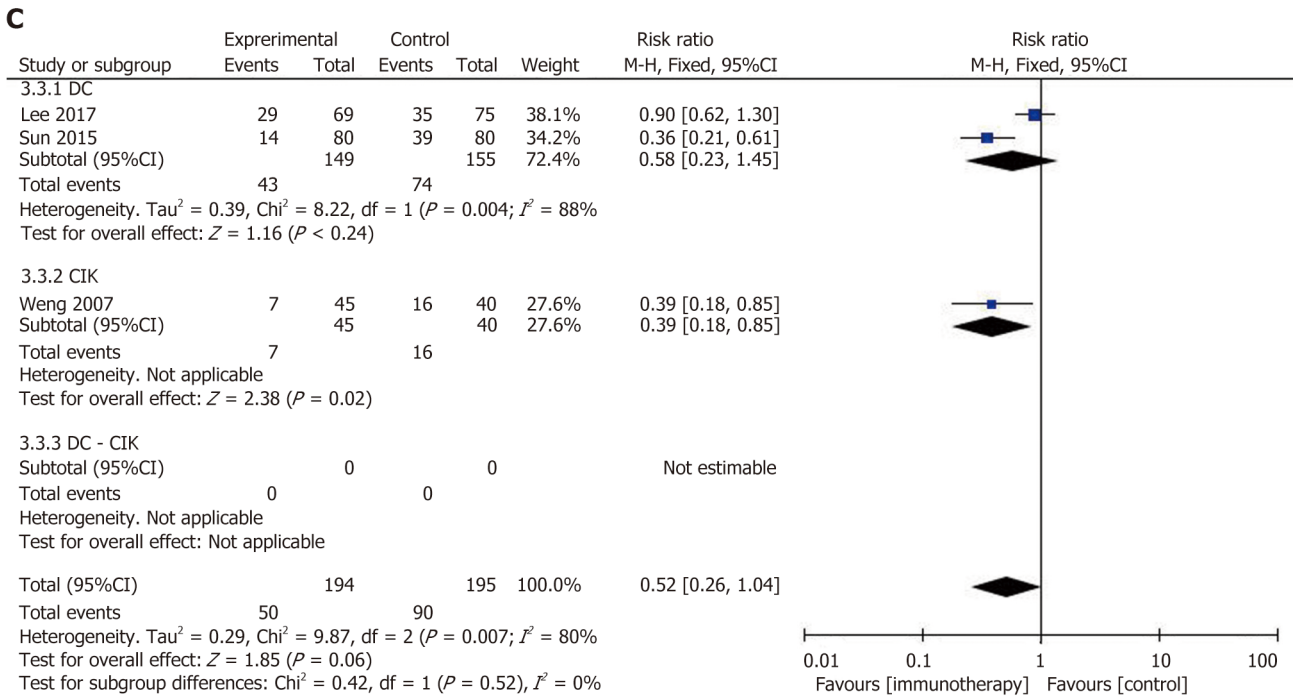


Figure 3 Comparison of 6-mo (A), 1- (B) and 1.5-yr (C) recurrence between immunotherapy groups and control groups. M-H: Mantel-Haenszel estimates; CI: Confidence interval.

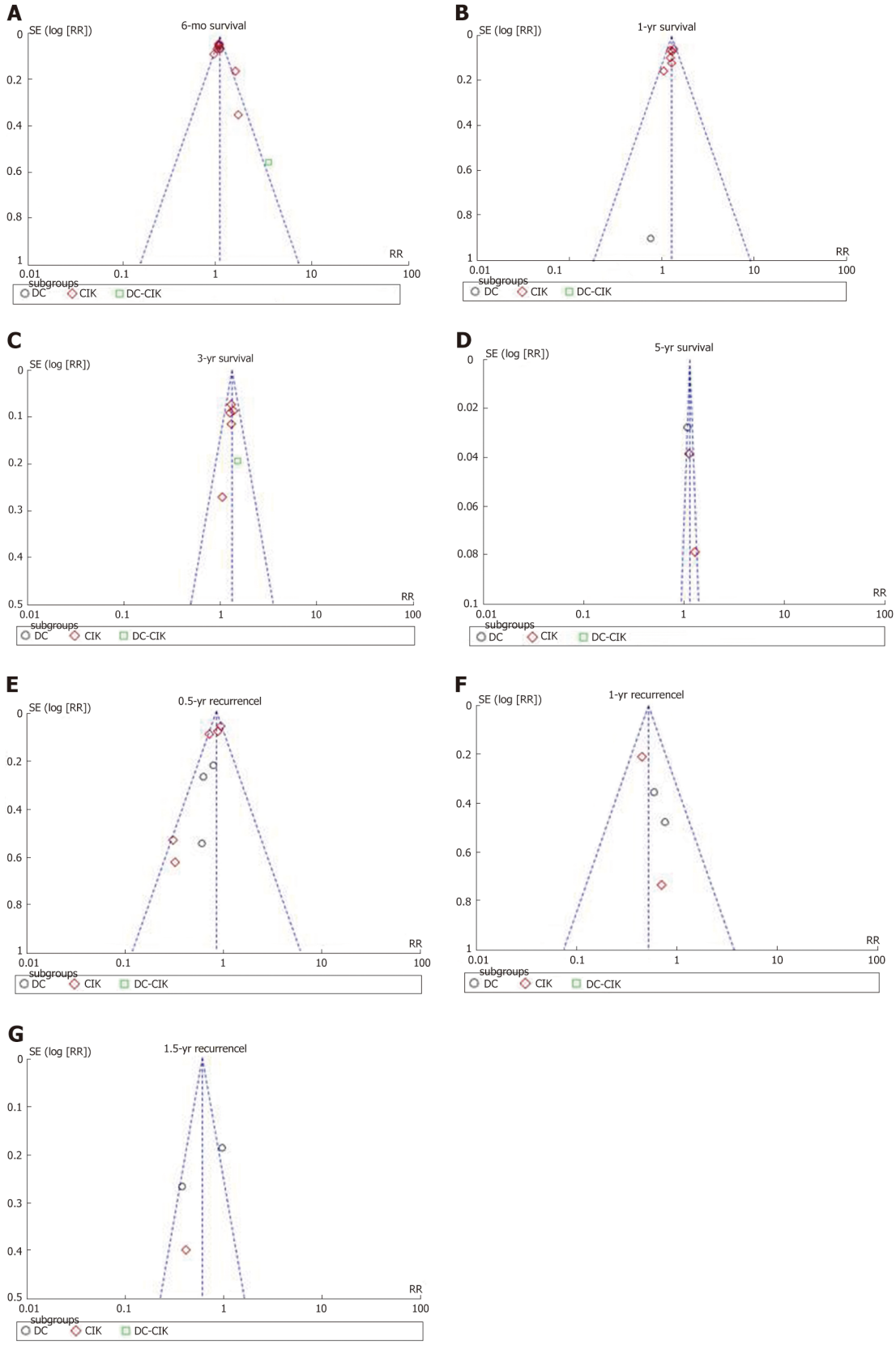


Figure 4 Funnel plots to detect publication bias. DC: Dendritic cell; CIK: Cytokine-induced killer cell.

ARTICLE HIGHLIGHTS

Research background

Hepatocellular carcinoma (HCC) has been revealed as the second most common cause of cancer-related deaths worldwide. The introduction of cell-based immunotherapy, including dendritic

cells (DCs) and cytokine-induced killer cells (CIKs), has brought HCC patients an effective benefit. However, the efficacy and necessity of cellular immunotherapy after different interventional therapy remain to be further explored.

Research motivation

Only patients with early to intermediate stage of HCC can benefit from curable interventions. Unfortunately, tumor recurrence within 5 years occurs even with curable treatment. As the introduction of immunotherapy has brought beneficial effects to HCC treatment, better strategies with combined interventions would help to improve the outcomes of HCC patients.

Research objectives

A systematic review and meta-analysis were performed in this study to investigate the efficacy of cellular immunotherapy, involving DCs, CIKs and DC/CIK combination therapy combined with different treatments of HCC.

Research methods

A literature search was performed on PubMed and Web of Science up to February 15, 2019. Long-term efficacy (overall survival and recurrence) and short-term adverse effects were investigated to assess the effectiveness of immunotherapy with DCs and/or CIKs. Review Manager 5.3 was used to perform the analysis.

Research results

A total of 22 studies involving 3756 patients selected by eligibility inclusion criteria were forwarded for meta-analysis. Combined with the conventional clinical treatment, immunotherapy with DCs and/or CIKs was demonstrated to significantly improve overall survival at 6 mo, 1 year, 3 years and 5 years. Recurrence rate was significantly reduced by cellular immunotherapy at 6 mo and 1 year. Adverse effect assessment addressed that immunotherapy with DCs and/or CIKs was accepted as a safe, feasible treatment.

Research conclusions

Combination immunotherapy with DCs, CIKs and DC/CIK with various routine treatments for HCC was evidently suggested to improve patients' prognosis by increasing overall survival and reducing cancer recurrence.

Research perspectives

This meta-analysis indicated that the combination of conventional therapy and the intervention of immunotherapy with DCs, CIK and DC/CIK could pave the way for a promising approach for HCC treatment. Further assessment of the efficacy of novel immune checkpoint inhibitors based on clinical trials will help us to better identify immunotherapy strategies to treat HCC.

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