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OPINION REVIEW

How to avoid overtreatment of benign colorectal lesions: Rationale for an evidence-based management

Marco Bustamante-Balén

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Abstract

Implementing population-based screening programs for colorectal cancer has led to an increase in the detection of large but benign histological lesions. Currently, endoscopic mucosal resection can be considered the standard technique for the removal of benign lesions of the colon due to its excellent safety profile and good clinical results. However, several studies from different geographic areas agree that many benign colon lesions are still referred for surgery. Moreover, the referral rate to surgery is not decreasing over the years, despite the theoretical improvement of endoscopic resection techniques. This article will review the leading causes for benign colorectal lesions to be referred for surgery and the influence of the endoscopist experience on the referral rate. It will also describe how to categorize a polyp as complex for resection and consider an endoscopist as an expert in endoscopic resection. And finally, we will propose a framework for the accurate and evidence-based treatment of complex benign colorectal lesions.

Key Words: Colorectal polyps; Endoscopic mucosal resection; Colorectal surgery

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Core Tip: Despite endoscopic treatment being of choice for the treatment of large benign colorectal neoplasms, many lesions are still being referred to surgery. Problems in identifying a polyp as complex to resect, too much self-confidence of the endoscopists, and the lack of a referral pathway may be causes underlying this situation. The organization of a structured referral network may be the main step to reducing the overtreatment of benign lesions. Decisive support from Medical Societies and Public Administration is warranted to set up this paradigm change.

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INTRODUCTION

The implementation of population-based screening programs for colorectal cancer (CRC) has led to an increase in the detection of large lesions with benign histology. For example, in a French study of a population screening program based on fecal occult blood testing, 5% of the polyps found were larger than 3 cm[1]. Many of these patients are asymptomatic and of intermediate age, and removal of the lesions should be performed with the goal of maximum efficacy (complete resections, few recurrences) and maximum safety (few adverse effects). Traditionally, most polyps considered "large" were biopsied and then referred to surgery for segmental resection of the colon. However, in recent years, with the improvement of endoscopes and the development of new techniques, endoscopic treatment can be considered the treatment of choice. However, too many benign lesions are still being referred to surgery, with associated morbidity and increased costs. In this review, we will justify the selection of endoscopic therapy as the treatment of choice, dig into the main causes for referring benign lesions to surgery, and propose an organizational solution for this situation. Most of the evidence that will be reviewed here focuses on endoscopic mucosal resection (EMR) because is the endoscopic technique of choice in most instances, while other endoscopic techniques [*e.g.*, endoscopic submucosal dissection (ESD) or full-thickness resection] are indicated for a more specific type of lesions.

WHY SHOULD ENDOSCOPIC RESECTION AND NOT SURGERY BE THE THERAPY OF CHOICE FOR THE TREATMENT OF BENIGN COLONIC LESIONS?

EMR can currently be considered the standard technique for the removal of benign lesions of the colon due to its excellent safety profile and good clinical results. Large series of patients, especially from the Australian endoscopic resection group, support this claim. Moss *et al*[2] performed a prospective evaluation of all patients referred for EMR of polyps ≥ 20 mm. In this cohort of particularly complex polyps, complete resection was achieved in a single session in 89.2% of patients with a recurrence rate of 20.4%. This recurrence was mostly minute and easily treated endoscopically. As for adverse effects, the same group reported a clinically significant bleeding frequency of 6.0%, of which only 44% required endoscopy and only one case required embolization[3]. The proportion of deep mural damage or perforation was only 3% and 0.6%, treated in all cases by endoscopic methods[4].

At least two meta-analyses confirm these results. The first, which evaluated the endoscopic management of lateral spreading tumors, and which analyzed separately EMR and DSE, reported a proportion of complete resections for the former of 99.5%, and a proportion of recurrences of 12.6%, most of which were manageable endoscopically. In terms of adverse effects, there was an aggregate proportion of perforation of 1.2% and bleeding of 9.6%[5]. In the second meta-analysis, the proportion of surgeries attributable to complications of EMR was less than 1%[6]. Therefore, we have an effective and safe endoscopic technique for the treatment of benign colon lesions.

The therapeutic alternative to endoscopic resection is surgery, which has classically been the treatment of choice. However, even with current techniques, surgery is not free of complications. The overall rate of adverse effects at 30 days is between 14% and 25% in the most modern series (Table 1)[7-10]. In general, the proportion of adverse effects is higher in open surgery, and as the age of the patient increases[7,8,10]. Surgery is also associated with a non-negligible risk of mortality which, although it is usually somewhat less than 1% (Table 1), in patients > 80 years of age can reach almost 3%[7].

No randomized studies are comparing both therapeutic strategies and such a study is unlikely to be performed due to ethical problems. One way to overcome this is the use of propensity score matching. Wickham *et al*[11] evaluated 95 patients referred because of endoscopically unresectable colorectal lesions and compared them to 190 propensity score-matched controls. Endoscopic resection was achieved in 66 (70%) of patients with a reduced hospital stay, a lower unplanned 30-day readmission rate, and fewer postoperative complications (4.2% *vs* 33.9%; $P < 0.001$) compared to surgery. Another attempt to make this comparison has been made using theoretical models. Ahlenstiel *et al*[12] compared the theoretical mortality of colon surgery, calculated using a proprietary Association of Surgeons of Great Britain and Ireland score, with the actual mortality from EMR of benign lesions in a cohort of 1,061 patients. While the theoretical mortality from surgery was 3.3%, there were no deaths in the first 30 days after EMR. The NNT to prevent one death was only 30. A recent Dutch study, using a microsimulation system and taking into account fatal complications of surgery, compared expert endoscopic resection of benign lesions *vs* laparoscopic surgery. Referral to an expert reduced from 2.1 surgeries for

Table 1 Main recent series on the morbidity and mortality of surgery for benign colorectal lesions

Ref.	Year	Country	Data source	N	Mortality (%)	Colostomy/ Ileostomy (%)	Major adverse event (%)	Readmission (%)	Surgical re-intervention (%)
Peery *et al* [7]	2018	USA	National Inpatient Sample[1]	12.732	0.7	2.2	14.0	7.8	3.6
Zogg *et al* [8]	2016	USA	National Inpatient Sample[1]	68.462[2]	-	-	14.7	-	1.0
de Neree *et al*[9]	2019	Netherlands	Systematic Review	139.897	0.7	-	24.0	-	0-8.9
Ma *et al* [10]	2019	USA	National Inpatient Sample[1]	262.843	0.8		25.3	-	-

[1]All-payer inpatient healthcare database.

[2]Overall colon surgery (not only colorrectal epithelial lesions): % of adverse events are specific for surgery of benign colorectal lesions.

benign polyps/1000 individuals to 0.2/1000 reducing also the number of deaths[13]. Some observational studies performed in Eastern countries and focused on ESD confirm that the latter has a shorter hospital length stay, an inferior 30-day readmission rate, and a lower complication rate[14,15].

Furthermore, endoscopic treatment is cost-effective compared to surgery. At least four studies in different countries and contexts have compared endoscopic resection with surgery in terms of cost-effectiveness (Table 2). All agree that endoscopic resection (EMR or ESD) is cost-effective compared to surgery, and this difference widens if the complications of surgery are taken into account[13,16-19]. The development of adverse effects after surgery has been associated with a 106% increase in the average length of stay and a 91% increase in the average cost of hospitalization[10].

Therefore, and this is reflected in the clinical practice guidelines[20,21], it seems clear that endoscopic treatment should be the first-choice treatment for benign colonic lesions, provided that quality standards are maintained[21].

WHAT IS THE SITUATION IN ACTUAL CLINICAL PRACTICE?

Several studies from different geographic areas agree that many benign colon lesions are still referred to surgery. In a French study conducted in a population-based screening program with fecal occult blood test, out of 4,251 patients with at least one polyp, 4.1% were referred to surgery[1]. In a study conducted in the USA, 47% of colorectal lesions sent for surgery were benign polyps[22]. In the aforementioned study by Zogg *et al*[8] the mean number of annual colectomies performed for benign pathology in the United States was around 22,000. A study of the British CRC screening program showed that, in the period from 2006 to 2009, 21.7% of the polyps sent for surgery were directly operated on, without a prior endoscopic attempt. Depending on the centers, the use of surgery as the first therapeutic option varied between 7% and 36%[23]. Finally, a recent Australian survey study showed that 16.7% of respondents would send directly to surgery a 45 mm benign polyp[24]. Saade *et al*[25], in a retrospective review of 144 patients with surgical resection for benign colorectal polyps found that 82% were referred for surgery without attempting an endoscopic resection. Of those, 22% had polyps < 2 cm, a size that should be resected en bloc by an average endoscopist[21].

But in addition, the rate of referral to surgery is not decreasing over the years, despite the theoretical improvement in endoscopic resection techniques. In a retrospective review of a national surgical database also in the United States, it was found that the incidence rate of surgery for benign polyps increased significantly over time, from 5.9 *per* 100,000 patients in 2000 to 9.4 *per* 100,000 patients in 2014, while during this same period the rate of surgery for CRC decreased. This increase was significantly greater in urban academic hospitals, which is just where one would expect it to decrease[26]. A Dutch study reviewed a national database of anatomic pathology reports looking for all cases of benign colon lesions removed by surgery. They showed that the ratio of the number of resections for benign lesions to the total number of colonoscopies performed was significant and remained constant over the last decade (2005-2015), ranging from 0.37 to 0.26[27].

Table 2 Cost-effectiveness studies, endoscopic therapy *vs* surgery

Ref.	Year	Country	Endoscopic technique	Design	Comparison	Costs analyzed	Results
Swan *et al*[19]	2009	Australia	EMR	Observational monocentric	Endoscopy *vs* surgery, Considering surgery without major complications	Direct costs including a 1-day hospital stay for EMR, Loss of utility not considered	EAC: $2051 pp, SAC: $9041
Jayanna *et al*[16]	2016	Australia	EMR	Observational multicentric	Endoscopy *vs* surgery, Considering surgery with and without complications	Direct costs including hospital stay and adverse events, 1st surveillance endoscopy	EAC: $4668 pp, SAC: $12720, If surgery 7.5% complications -> SAC: $45530
Law *et al*[17]	2016	USA	EMR	Decision analysis tree (hybrid Markov model)	Endoscopy (resection + surveillance, surgery if recurrence at 12 mo) *vs* laparoscopic surgery, Considering complications in both arms	Direct costs, Loss of utility considered, QALY, Sensitivity analysis	EAC: $5570 ppEndoscopy QALY: 9.64, SAC: $18717 pp, Surgery QALY: 9.58, Laparoscopy is cost-effective if complete EMR < 75.8%, EMR adverse events rate > 12% and laparoscopy cost < $14.000
Dahan *et al*[18]	2019	France	ESD	Observational monocentric	Endoscopy *vs* surgery, Considering complications in both arms	Direct costs including hospital stay and endoscopy costs	EAC: €3190, SAC: €8490
Buskermolen *et al*[13]	2022	Netherlands	Non-specified	Microsimulation screening analysis (MISCAN-colon)	Surgery *vs* attempted removal by an expert endoscopist, Considering complications in both arms	Direct costs, Loss of utility considered, QALY, Sensitivity analysis	EAC: €60.200, SAC: €72.700, Endoscopy QALY: 33.1/1000 individuals, Surgery QALY: 32.9/1000 individuals

EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosa dissection; QUALY: Quality adjusted life years; EAC: Endoscopy average cost; SAC: Surgical average cost; pp: Per patient.

WHAT IS THE MAIN REASON THAT BENIGN LESIONS ARE STILL BEING SENT DIRECTLY FOR SURGERY? THE ENDOSCOPIST FACTOR.

Le Roy *et al*[1] evaluated different variables related to referral to surgery. Size, location, and histology (villous component and high-grade dysplasia) were influential. The most relevant factor was size > 20 mm. These data have been confirmed in a recent meta-analysis, in which the most frequent causes of referral to surgery were polyp location (right colon), size (median size = 4 cm), and sessile morphology [9].

However, the assessment of the relative importance of these factors when deciding whether to perform endoscopic or surgical resection is highly subjective and dependent on the endoscopist. In fact, in the study by Le Roy *et al*[1] great variability was found among endoscopists when referring the patient to surgery, a difference that was maximal for polyps > 20 mm (0 to 46.6%). These results were confirmed in a study in which 154 endoscopists of different expertise (residents, gastroenterologists, surgeons, and experts in polyp resection) responded to a survey on how to treat 6 polyps presented in video format. Endoscopists specializing in complex resections referred the patient to surgery at a significantly lower percentage than other specialists (3.1% *vs* 13.3% non-specialists endoscopists *vs* 17.2% surgeons). In the univariate analysis that took into account the size of the polyp, its location, the patient's ASA, and the type of specialist, the fact that the endoscopist was not a specialist in the resection of complex polyps was the only variable related to the probability of referring the patient to surgery [odds ratio (OR) 4.93, 95%CI (1.5-16.26)][28].

This variability in clinical practice has direct practical consequences. A retrospective review of the Dutch pathology registry reviewed the medical reports of patients referred for surgery for benign polyps over 9 years. Three expert endoscopists reviewed the records to assess whether the patient could have benefited from endoscopic treatment. The conclusion was that 73% of the cases could have been treated endoscopically. This referral to surgery as a primary elective treatment was more frequent in county hospitals than in tertiary referral centers[29]. In other words, the endoscopist is perhaps the most influential factor in whether a patient is referred for surgery, and experience in complex resections seems to be the fundamental characteristic.

IS IT EFFECTIVE TO REFER THE PATIENT TO AN ENDOSCOPIST WITH EXPERTISE IN COMPLEX ENDOSCOPIC RESECTIONS?

The possibility of reducing surgeries if the patient was referred to an expert endoscopist has also been demonstrated in practice. In one study, 58 patients referred to surgery for colorectal polyps were collected. An expert endoscopist re-evaluated these lesions in a new colonoscopy to decide whether endoscopic resection was possible. Of these 48 could be resected endoscopically although 5 of them underwent surgery later either because of malignancy in the specimen (4 cases) or recurrence (1 case). In any case, surgery could be avoided in 43 (74.1%)[30]. Other studies of similar design agree that surgery is avoidable in 30-70% of cases when the polyp is reviewed by an expert endoscopist, including up to 26% of lesions with previously attempted resection[31-33].

WHY IS AN EXPERT ENDOSCOPIST MORE EFFECTIVE?

The main advantage of an experienced endoscopist when removing complex lesions is that he or she will have a higher proportion of complex resections with a lower frequency of adverse effects.

Few studies are comparing the results of EMR in terms of efficacy according to the experience of the endoscopist. The St. Marks group evaluated the proportion of successful resection between a group of expert and non-expert endoscopists. Experts were successful in 76% of cases while non-experts were successful in only 40%[34]. A retrospective study showed that the performance of resection by an expert endoscopist was protective against incomplete resection in the presence of other risk factors for incomplete resection [adjusted OR 0.13, 95%CI (0.04-0.41)][35]. The CARE study demonstrated that the rate of incomplete resection in polyps that, in the judgment of the endoscopist, were assumed to be completely removed was high (10.1%), increased with polyp size, and was highly dependent on the endoscopist. These findings suggest that technical skill in complex resections is not universal[36].

The experience of the endoscopist is also a key factor for adverse effects. In a study of 97,091 colonoscopies performed on an outpatient basis, the OR for bleeding or perforation increased significantly when the endoscopist performed fewer than 300 colonoscopies per year[37]. In a similar study of 24,509 endoscopies, the complication rate was significantly higher for endoscopists performing fewer than 200 procedures per year [RR 2 95%CI (1.1-3.7)][38]. Finally, a study of 2,315,126 colonoscopies confirmed that endoscopists performing fewer than 300 colonoscopies per year had a higher rate of bleeding and perforation[39].

HOW DO WE KNOW THAT AN ENDOSCOPIST IS AN EXPERT? THE EGO OF ENDOSCOPISTS.

Some of the studies evaluating the rate of surgery for benign polyps have found that this rate is higher in urban teaching hospitals, hospitals that often have experts or units specialized in endoscopic resection[7]. There may be too much self-confidence in the endoscopist (*e.g.*, "if I cannot resect this lesion, nobody can") or there may be some feeling of shame in referring a lesion to a colleague.

The endoscopists' perception of their expertise is often not supported by objective criteria. This fact was elegantly highlighted in a study in which 268 surveys were conducted among endoscopists asking them, among other things, about their experience in resection and their surgical referral practices. Eighty-one (30%) of them considered themselves capable of performing complex resections on lesions that could perfectly well have been referred to surgery. However, of this group of "experts" 17% had never removed a polyp > 5 cm and 32% did not perform more than 20 EMRs per year. In other words, a significant number of endoscopists considered themselves experts in resection when there was no objective evidence of this. And this had consequences for patient management because endoscopists who considered themselves non-experts tended to send patients to another colleague, while "experts" more frequently sent them to surgery (26% *vs* 68%)[40].

Inappropriately mischaracterizing oneself as an expert endoscopist directly affects patient management in three key ways: (1) The endoscopist will initiate a resection that he or she cannot complete, and complications are possible; (2) If resection is not attempted the patient is more likely to be sent to surgery than a more expert colleague; and (3) As a consequence of the previous two, the patient is more likely to be incorrectly sent to surgery.

The definition of some objective criteria to classify an endoscopist as an expert in resections could help in this situation, reassuring the less-experienced endoscopist to refer the lesion to a better-prepared endoscopist. However, there are no established criteria to identify the expert endoscopist in performing EMR, perhaps due to the lack of structured training for this technique.

WHAT LESIONS SHOULD BE REFERRED AND TO WHOM? HOW TO TRANSFORM SUBJECTIVITY INTO OBJECTIVITY

As we have seen, the assessment of the difficulty of resection of a particular polyp depends primarily on polyp factors (size, morphology, location, suspicion of submucosal invasion, *etc.*) that may seem rather subjective in their evaluation. It seems logical, therefore, to develop systems that are as objective as possible to define which polyp should be sent to an expert endoscopist, in such a way as to help endoscopists of varying degrees of experience to make the decision. On the other hand, it seems necessary to have criteria for evaluating endoscopists to define, as objectively as possible, what is an expert endoscopist, aimed to easily identify referral specialists. Finally, the expertise of the individual endoscopist is not enough. His or her work environment must allow for comprehensive treatment of benign colon lesions, with the use of different resection techniques depending on the case, and must have sufficient casuistry to maintain the skills acquired. In this section, we will review these three sides of the management of large colorectal lesions: The complex polyp, the expert endoscopists, and the reference endoscopy unit.

The "complex" polyp

To avoid or at least reduce individual subjectivity in the assessment of the difficulty of resection, objective evaluation criteria are necessary. A group of experts, following the Delphi methodology, defined a score to classify the theoretical difficulty in the resection of colon polyps. This score ("SMSA" scoring system) has four parameters (Site, Morphology, Site, and endoscopic Access), and assigns different scores to the values adopted by each one. Thus, a polyp > 4 cm, with a flat morphology, located in the right colon, and with difficult access obtains the highest score (17 points). All polyps scoring > 12 points are considered level 4, and appropriate for truly expert endoscopists[41].

The British Society of Gastroenterology (BSG) suggests other lesion´s objective features that anticipate a complex resection, grouped into three areas: Increased risk of malignancy evidenced by optical diagnosis, increased risk of incomplete resection, and increased risk of adverse effects (Table 3). Notably, the experience of the endoscopist is included as a criterium for defining a complex polyp because of an increased risk of adverse events[42]. The definition of a complex polyp, therefore, involves a judicious and sensible evaluation of the endoscopist´s expertise.

The categorization of a polyp as complex involves more than the difficulty in its removal. The management of a complex polyp may also need, to some extent, and depending on the lesion´s characteristics, the need for expert, interdisciplinary management. In this sense, the European Society of Gastrointestinal Endoscopy (ESGE) recommends sending the lesion to be evaluated in an expert center, besides the aforementioned criteria, when superficial submucosal invasion is suspected[21].

The "expert" endoscopist

The level of experience required of endoscopists to resect polyps is not objectively defined. The ASGE guidelines indicate that all endoscopists should be able to resect pedunculated or sessile polyps < 2 cm [43], but resection of complex polyps requires special skill, specific learning, and experience, and it seems unreasonable to expect this from all centers[44].

The most objective criterion could be the number of resections performed. However, this particular number has not been defined yet. Several studies place the experience necessary to perform EMR with adequate quality standards between 100 and 125 resections[45,46]. Other authors, based on a retrospective study of a new EMR unit for 4 years, suggest a number of 30 EMRs per year, but the SMSA level of their lesions was not described [47]. This figure seems a bit low when dealing with SMSA level 4 Lesions. The BSG broadly suggests that the number of resections per year should be enough to maintain acceptable quality and safety standards, but also indicates that there is no evidence to recommend a specific figure[42]. Regarding ESD, there is also a high variability in the reported number of cases needed to achieve proficiency ranging from 20 to 250 cases[48]. To maintain proficiency, the ESGE curriculum recommends performing at least 25 cases per year[49].

Some more objective methods to evaluate polypectomy competency have been developed, like the Direct Observation of Competence Skills (DOPyS). This instrument assesses several items, like optimal polyp position, determining the full extent of the lesion, polypectomy technique, *etc.* Using this tool Duloy *et al*[50] described significant variation in polypectomy competency rates (30% to 90%) with rates decreasing for larger polyps. However, it has not been designed specifically for EMR. The BSG has proposed auditable indicators to assess the ability of endoscopists to perform EMR, focusing on efficacy (% recurrences), safety (% complications), and annual case volume (Table 3)[42].

The implementation of structured learning tools or courses could help to evaluate who may be competent in endoscopic resection techniques. *In vivo* and virtual tools have been described for EMR and ESD[51,52], and a formal curriculum for ESD has been developed by the ESGE[49]. However, there is not a similar curriculum for EMR training, which has essentially been limited to that obtained during residency and has repeatedly proven to be insufficient[53].

Table 3 Objective parameters for assessing lesions, endoscopists, and units

Complex polyp	Expert endoscopist	Reference endoscopy unit
SMSA score ≥ 12 (Level 4)[41]	BSG criteria[42]	BSG criteria[42]
BSG criteria[42]	500 independent colonoscopies	Ensure that endoscopists undertake a sufficient number of procedures a year to maintain acceptable standards[4]
Increased risk of malignancy	100-125 EMR to obtain competence	
Kudo´s pit pattern V	A non-defined number[1] of EMR procedures to maintain competence	
Paris 0-IIc/0-IIa+IIc		Time from referral to definitive management: < 8 wk
LST-NG/LST-Gm (dominant nodule)	Fulfilling key performance indicators	
NICE 3/Sano III	Presence of recurrence/residual polyp at 12 mo < 10%	Geraghty *et al*[40]
Increased risk of incomplete resection/recurrence		Provided endoscopy list time for the additional workload with a dedicated list
Size ≥ 40 mm	EMR perforation rate: < 2%	
Difficult location (ileocecal valve, appendix, diverticulum, dentate line)	Post-polypectomy bleeding rate: < 5%	Staff to include at least two endoscopists that can cover each other and endoscopy nurses with training in complex polypectomy
Within an inflamed segment of the colon	DOPyS[2]	
Prior failed resection attempt	ESGE[3] curriculum for optical diagnosis[59]	
Non-lifting sign	Assessing competence: ≥ 80 % accuracy for identifying submucosal invasion in large (≥ 20 mm lesions), Maintaining competence: *in vivo* audit and review of at least 10 large (≥ 20 mm) lesions within a year	Equipment: including necessary snares and hemostatic devices
Increased risk of adverse events		Surgeons for discussion in the MDT and case of operative treatment of adverse events
Cecum		
Endoscopist´s expertise		Robust referral system including administrative staff support and tools for virtual MDT
ESGE criteria[21]		
Difficult location or poor access (ileocecal valve, periapendicular, anorectal junction)		
Prior failed resection attempts		
Non-lifting sign		
SMSA level 4		

[1]Enough to maintain quality standards.
[2]Direct Observation of Competence Skills (not specific for EMR).
[3]For achieving competence in optical diagnosis of early colorectal cancer.
[4]Review in conjunction with other key performance indicators.
BSG: British Society of Gastroenterology; EMR: Endoscopic mucosal resection; LST-NG: Non-granular lateral spreading tumor; LST-Gm: Granular mixed lateral spreading tumor; NICE: NBI International Colorectal Endoscopic classification; ESGE: European Society of Gastrointestinal Endoscopy; MDT: Multi-disciplinary team; DOPyS: Direct Observation of Competence Skills; SMSA: Site, Morphology, Size, Access.

Moreover, for increasingly larger polyps, with flat morphology, in difficult locations, or patients with previous colon pathology such as inflammatory bowel disease, the endoscopist must also master alternative mucosal resection techniques such as the underwater technique, or fragmented cold loop resection, and know how to choose between them by changing the initial resection plan. Strategies to decrease the recurrence rate, like margin ablation, margin marking, or hybrid argon plasma coagulation [54-56] should be mastered as well. Finally, an endoscopist specializing in endoscopic resection of larger lesions must also know and apply optical diagnosis (use of NICE and JNET classifications, use of dyes, use of magnification, *etc.*) to identify those that, due to a higher probability of superficial submucosal invasion, require an en bloc resection, using ESD or full-thickness resection[20,57]. And also, those that, due to a high probability of deep submucosal invasion, must be surgically removed[58]. Following, for instance, the BSG guidelines, these skills are needed to correctly classify a polyp as complex (Table 3) [42]. Mastering optical diagnosis also needs proper training and practice to obtain and maintain competence[59].

The "reference" endoscopy unit

It seems that the number of procedures is the single most important factor influencing on efficacy and safety results of an endoscopy unit specialized in complex resections. In the aforementioned Australian study on risk factors for post-polypectomy bleeding, the unit (one that had performed fewer than 75 procedures) was directly related to the likelihood of immediate post-polypectomy bleeding [adjusted OR 3.78 (2.35-6.10)] and to bleeding occurring beyond the first 48 h[60]. And immediate post-polypectomy bleeding was related to the probability of recurrence at the first endoscopic control. Other studies describe a lower rate of complications in colonoscopies performed in a hospital center than in an outpatient clinic[39]. It thus appears that units that accumulate a larger number of cases are more effective in the treatment of complex colon lesions. This has also been shown in studies on newly developing EMR units, in which the rate of complications decreased as experience time was gained[47]. The frequency of SMSA level 4 Lesions in a single institution is unlikely to be enough to maintain competency, therefore a centralized referral system seems advisable[40].

But in addition to the experience and casuistry of the units, the adequate management of complex colon lesions requires adequate infrastructure. High-definition endoscopes that allow precise optical diagnosis, electrosurgical units with automatic microprocessors, CO_2 insufflation, specific pumps for lavage channels, *etc.*[21]. They also need the availability of a variety of resection devices (snares, knives, injection substances, hemostatic, *etc.*) allowing switching resection techniques and dealing with complications. Resections of complex polyps lengthen the procedure time beyond that required for a conventional colonoscopy[61], prolongation which is closely related to the size of the polyp in question. Therefore, the unit will have to have the facility to adjust the citation slots to the performance of longer and more complex procedures. Finally, the work of this kind of unit must be integrated into a background with experienced surgeons, a multidisciplinary team for the management of complex lesions[40], and all the infrastructure (computed tomography scanning, *etc.*) to handle possible adverse events.

The BSG has also proposed measurable domains for accrediting Endoscopy Units for performing EMR (Table 3). Regarding ESD, the American Society of Gastrointestinal Endoscopy recommends setting up an "ESD cart" with the necessary equipment for the procedure and the management of adverse events. The presence of experienced nurses and technicians is also addressed[48].

IS IT TRULY EFFICIENT IN REAL PRACTICE TO REFER COMPLEX POLYPS TO EXPERIENCED UNITS?

We have learned that an appropriate referral of complex polyps to an expert endoscopist increases the rate of successful endoscopic treatment and reduces adverse events compared to surgery. We have also learned how a referral endoscopy unit should be to ensure efficacy. How has this been translated onto clinical practice?

The Australian group was the first to demonstrate the efficacy of a referral unit for the treatment of large colorectal lesions. Out of 174 patients referred for 193 complex polyps, 90% avoided surgery with a procedural success of 95% excluding those patients with invasive cancer[19]. Another Australian retrospective study comparing the surgical rate of benign colorectal lesions before and after the introduction of a specific EMR service in a tertiary referral center showed a 56% reduction in the number of patients referred to surgery[62]. More recently, in France, a study evaluated the evolution of surgical management of benign polyps > 2 cm after the implementation of a regional referral network for the management of these lesions. This regional care network included two specialized endoscopists in the referral center with direct access by e-mail or by phone to all general gastroenterologists in the region and with twice-a-year regular meetings with general gastroenterologists. The surgical management rate of benign lesions decreased significantly after the implementation of the referral network from 14.6% in 2012 to 5% in 2017[63]. Similarly, in the Northwest of the Netherlands, a reference panel of expert endoscopists for the general endoscopist to consult was organized. Eleven centers participated and 88 patients were evaluated by the panel. Overall, 43.2% of consulting endoscopist changed their initial management strategy after consultation, and in 56 cases (63.3%), the patient was referred to another endoscopy center[64].

In conclusion, setting up a referral system for the management of complex polyps is efficient and translates into immediate clinical advantages.

HOW TO SET UP A REFERRAL ENDOSCOPY UNIT FOR THE MANAGEMENT OF COMPLEX POLYPS: PRACTICAL TIPS AND AN ORGANIZATION PROPOSAL

To achieve the objective of an adequate and comprehensive treatment of large benign colon lesions, several actors must be involved: The Administration, the Scientific Societies, the Units themselves, and

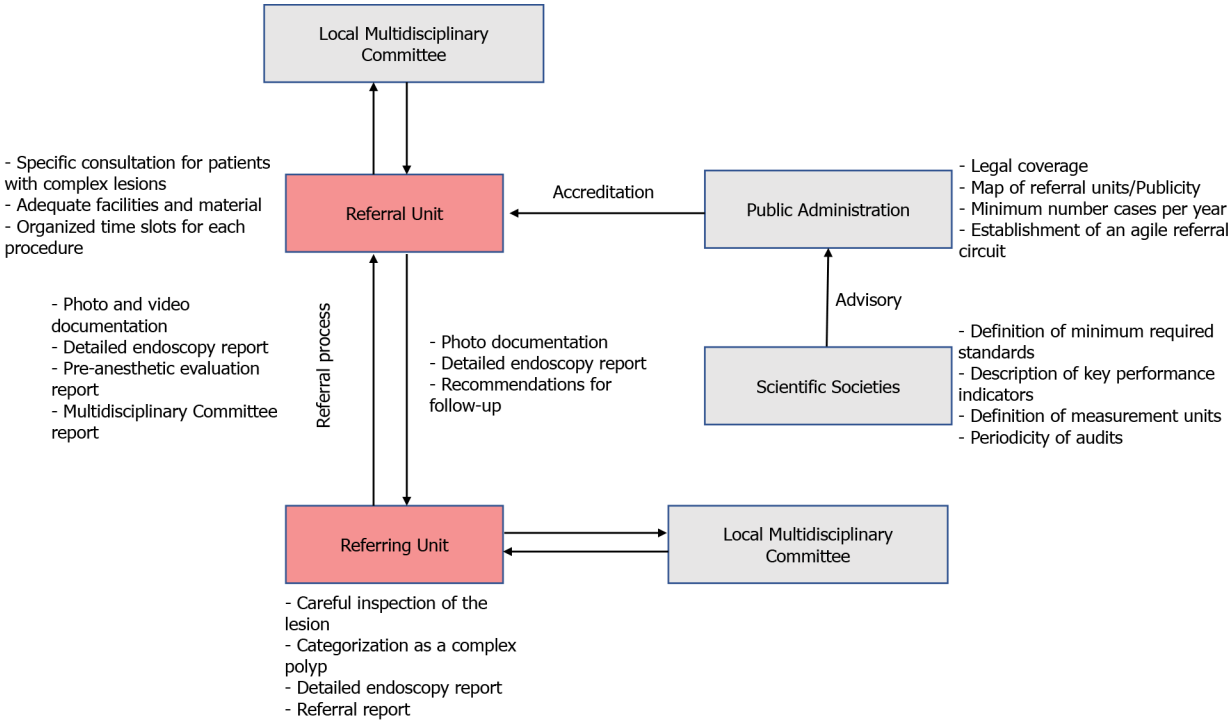

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Figure 1 Organizational proposal for the management of complex benign colorectal lesions.

finally the referring endoscopists (Figure 1).

One side of the referral network is the referral endoscopy unit. Having established the main characteristics that a referral unit must have to be considered as such, a certification system should be put in place. For instance, using criteria similar to that of the BSG plus others adapted to the specific background, periodical audits of the organization and key performance indicators should be performed. The local Endoscopy Societies should collaborate in the design of the certification protocol (definition of key indicators, measurement units, audit´s periodicity, *etc.*). An example of this kind of collaborative effort, although not applied to complex resections, is the Qualiscopia initiative in Spain, which aimed to monitor and certify endoscopy units and endoscopists in quality in colorectal screening colonoscopy [65]. Ideally, the endoscopy unit has to establish an internal Quality Management Program, including the definition of a system for recording staff´s initial competency and continued competency on an annual basis. Working together with the referral unit there should be a multidisciplinary team in place, made up of surgeons, oncologists, radiologists, and every specialist that could be involved in the management of complex polyps, especially when facing a deep submucosal invasion or dealing with adverse events. Finally, the referral unit should have enough administrative staff to handle the communication and documentation workload from and to the referring units. Clean communication systems should be established (e-mail, telephone, videoconference, *etc.*).

The Administration should, in agreement with the Societies, should give legal cover to the concept of Referral Unit, should establish a map of referral units according to population needs, ensure a minimum number of cases per unit to maintain competence, and should participate in the establishment of an agile regional referral circuit[21,42]. It is the Administration that should make available for all possible referring physicians a list with the accredited Units.

The other face of the referral network is the referring endoscopist. These endoscopists have to carefully evaluate and characterize the lesion using the usual classifications (NICE, Paris, *etc.*), categorize it as a "complex lesion" and decide if he/she can resect it. If not, the lesion should not be biopsied (unless an invasive carcinoma is suspected), and a tattoo should be placed not too close to the lesion[66]. Several pictures and videos should be taken. All this information should be provided in a detailed and structured endoscopy report. Ideally, a Multidisciplinary Committee (including at least an endoscopist and surgeons) should evaluate the patient and make a report that should be incorporated into the referral report.

The referral process should be detailed including clinical and administrative data from the patient, photo and video documentation of the lesion, a detailed endoscopic report including size, location, and morphology of the lesion, if biopsies were taken or if there was any resection attempt. The reason for referring the lesion should also be described. In correspondence, once the lesion has been treated in the referral unit, a thorough report of the applied treatment should be done, again with photo documentation. The technical result of this treatment (success/failure) should be provided. Finally,

recommendations for patient follow-up should be attached to the report. It is mandatory to maintain fluid communication between referring and referral units during the therapeutic process. Al the steps back and forth in the referral process should be subjected to the Quality Management Program and should be auditable.

CONCLUSION

Endoscopic resection is the treatment of choice for large colorectal lesions. However, overtreatment is still an important issue in many countries. Organizing a network of specialized endoscopy units in complex resections seems to be the main approach to tackling this situation. This development should be accompanied by the organization of an accreditation system and a Quality Management Program, a process in which endoscopy units, endoscopists, Scientific Societies, and the Public Administration should be involved.

FOOTNOTES

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REVIEW

Mucosal imaging in colon polyps: New advances and what the future may hold

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Abstract

An expanding range of advanced mucosal imaging technologies have been developed with the goal of improving the detection and characterization of lesions in the gastrointestinal tract. Many technologies have targeted colorectal neoplasia given the potential for intervention prior to the development of invasive cancer in the setting of widespread surveillance programs. Improvement in adenoma detection reduces miss rates and prevents interval cancer development. Advanced imaging technologies aim to enhance detection without significantly increasing procedural time. Accurate polyp characterisation guides resection techniques for larger polyps, as well as providing the platform for the "resect and discard" and "do not resect" strategies for small and diminutive polyps. This review aims to collate and summarise the evidence regarding these technologies to guide colonoscopic practice in both interventional and non-interventional endoscopists.

Key Words: Colonoscopy; Colorectal cancer; Mucosal imaging; Chromoendoscopy; Polyp surveillance; Polyp characterization

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Core Tip: Advanced mucosal imaging enhances polyp detection and characterization. This detailed review summarises existing advanced mucosal imaging technologies to guide everyday colonoscopic practice for interventional and non-interventional endoscopists.

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INTRODUCTION

Colorectal cancer (CRC) accounts for 10% of cancer incidence and is the third leading cause of cancer-related death worldwide[1]. Whilst CRC incidence and mortality are increasing globally, there is now tangible evidence of the evolving efficacy of screening programs in developed countries including Australia, the United States, Iceland, New Zealand and Japan, where there have been improvements in both CRC incidence and mortality[2,3]. While these decreases are multifactorial and partly a result of lifestyle modification (reduction in smoking, weight loss, dietary changes), the implementation of population CRC screening programs has been integral to the prevention and early detection of CRC[4,5].

CRC develops through a well-documented adenoma-carcinoma cascade consisting of multiple differing pathways. Although underlying genetic mutations are diverse and heterogenous, most CRCs arise as either traditional tubular adenomas or serrated adenomas. Eventually these adenomas acquire additional carcinogenic mutations sufficient to develop invasive potential[6]. This sequence forms the basis of colonoscopic screening and surveillance programs. Not only can cancers be detected at an early stage where curative and non-invasive treatment is possible, but in many cases these pre-cancerous adenomas can be resected prior to their differentiation into carcinomas with invasive potential.

Unfortunately, interval CRCs still develop in patients who have undergone appropriate colonoscopic screening, accounting for 4.8%-7.9% of all CRCs[7-11]. Given that most adenomas take an estimated 5-15 years to develop into CRC, these interval cancers likely represent adenomas missed at the time of colonoscopy[12]. In fact, a 2019 meta-analysis found miss rates for adenomas to be as high as 26%[13]. Studies have consistently demonstrated that location in the proximal colon leads to an increased chance of missed adenomas, with interval cancers more than twice as likely to be proximally located[11]. Multiple factors contribute to this risk, as proximally located polyps are more likely to be flat, more likely to be sessile serrated polyps, more dysplastic whilst smaller and less likely to be hyperplastic polyps without malignant potential[14-16].

While certain polyp-related factors contribute to the likelihood of missed adenomas, overall adenoma detection rates (ADRs) are also highly operator-dependent. For example, a retrospective propensity-score matched study demonstrated an ADR of 44% for "high-ADR endoscopists" *vs* 26.9% for "low-ADR endoscopists" in the same Japanese screening population[17]. In this study, "high-ADR endoscopists" were more likely to detect proximal, non-protruding and high-risk adenomas. It is therefore not surprising that studies have demonstrated an inverse correlation between endoscopists" ADR and interval cancer development, with each 1% increase in ADR resulting in a 3% reduction in interval cancer risk[18,19]. Kaminski *et al*[19] also demonstrated an increase in interval cancer development in endoscopists with an ADR < 20%. Accordingly, societal guidelines recommend a minimum ADR of 25% (20% in women, 30% in men) as a means of ensuring quality control among colonoscopists[20]. More recently, the mean number of adenomas detected during colonoscopy has been raised as a possible alternative quality indicator, as the number of adenomas detected directly impacts surveillance intervals. Denis *et al*[21] found that even endoscopists with an ADR of more than 35% had considerable variation in mean adenoma detection over 42817 surveillance colonoscopies, from 0.36 to 0.98. The adenoma miss rate has also been demonstrated to vary considerably between high ADR endoscopists, instead correlating strongly with adenomas detected per colonoscopy[22].

Given the heterogeneity among proceduralists and the ongoing prevalence of interval CRCs, multiple add-on devices and techniques have been developed to increase mucosal visualisation and reduce adenoma miss rates. A 2020 network meta-analysis demonstrated that add-on devices such as "Endocuff vision" and techniques such as water-immersion colonoscopy do improve adenoma detection [relative risk (RR) 1.53 and 1.41 respectively] however they require additional equipment and cost while often increasing procedure times[23]. The addition of a transparent cap attached to the tip of the colonoscope has been demonstrated to improve adenoma detection while also reducing caecal intubation time[24-26]. However, a 2012 meta-analysis found the impact of these measures to be small, with a RR of 1.08 for adenoma detection and a mean 0.64 min reduction in caecal intubation time[27]. In the context of expansive population screening programs, small changes in equipment costs and procedure times have a considerable impact on a larger scale.

Advanced mucosal imaging techniques function by either improving image definition, application of dyes/altering the light source to enhance certain tissue features, digitally enhancing images in real time, or by providing "alerts" to the proceduralist for abnormal findings detected by artificial intelligence (AI). In doing so, these technologies aim to improve detection and characterisation of polyps without increasing equipment costs. This review aims to consider and summarise the numerous available advanced imaging technologies and examine their efficacy in both polyp detection and polyp characterisation. Whilst this is not a formal systematic review, it has been based largely on a structured interrogation of existing literature using Pubmed and Embase, with abstracts screened for relevance and reference lists searched for additional pertinent studies.

POLYP DETECTION

Standard and high-definition white light imaging

White light imaging (WLI) is the original unenhanced form of endoscopic imaging. Standard definition (SD-WLI) endoscopes produce a signal of up to 100000 to 400000 pixels, compared to high-definition (HD-WLI) endoscopes which produce from 850000 to more than 1 million pixels[28]. Despite this considerable improvement in image quality, studies comparing HD-WLI to SD-WLI have found an only marginal benefit in adenoma detection, with a 2020 meta-analysis of 6 randomised-controlled trials (RCTs) involving 4594 patients finding an ADR of 40% for HD-WLI *vs* 35% for SD-WLI (RR 1.13, P = 0.001)[29-31]. However, various studies have demonstrated a more significant increase in detection of flat adenomas (8.2%-9.5% *vs* 2.4%-3.8%), right sided adenomas (34% *vs* 19%) and sessile serrated polyps (RR 1.55, P = 0.03) with HD-WLI[29,31,32]. In the context of inflammatory bowel disease (IBD) where dysplasia detection is notoriously difficult, HD-WLI leads to increased likelihood of dysplasia on targeted biopsies, with an adjusted prevalence ratio of 2.99 (CI 1.16-7.79) in one 2013 study[33]. In fact, Krugliak *et al*[34] described 36 patients who underwent colectomy for dysplasia in IBD found using HD-WLI colonoscopy, in which no metachronous lesions were discovered that had not been detected endoscopically. While the overall benefit in adenoma detection may be marginal, the improved detection of high-risk, flat, right sided lesions, along with the fact that HD-WLI is now widely available, has led to almost universal uptake of HD-WLI in screening colonoscopy.

Chromoendoscopy

Chromoendoscopy involves topical application of dyes to enhance mucosal characterisation and improve detection of pathologic lesions. For adenoma detection during colonoscopy, the most commonly used dye is methylene blue, which is rapidly absorbed into healthy colonic mucosa and more slowly absorbed in dysplastic tissue[35]. More recently, chromoendoscopy using acetic acid has been described, acting as a mucolytic agent as well as increasing mucosal surface opacity[36].

Multiple studies have demonstrated the efficacy of chromoendoscopy for neoplasia detection (particularly proximal serrated lesions) during screening and surveillance colonoscopy, with a 2016 Cochrane review (7 studies, 2727 participants) finding an odds ratio (OR) of 1.53 for detection of at least one neoplastic lesion[37,38]. However, the incremental benefit in many of these studies has been marginal and not associated with any increase in detection of advanced adenomas or larger polyps[39,40]. The strongest evidence for the benefit of chromoendoscopy has been for detection of dysplasia in the IBD population. Compared to SD-WLI, multiple meta-analyses have demonstrated the superiority of chromoendoscopy, with a RR of up to 2.05 for dysplasia detection[41,42]. However, the utility of chromoendoscopy in IBD has become more controversial as more recent studies have not demonstrated a difference between chromoendoscopy and HD-WLI[41,43,44].

Chromoendoscopy has been shown to improve dysplasia detection in other high-risk populations, particularly in those with an increased risk of flat, right-sided lesions. A 2019 tandem study comparing HD-WLI and chromoendoscopy with indigo carmine in patients with serrated polyposis syndrome found a higher additional ADR (39% *vs* 22%, P < 0.001) in the chromoendoscopy group[45]. In hereditary non-polyposis colon cancer (HNPCC), a 2019 meta-analysis demonstrated improved adenoma detection with a relative risk (RR) of 1.53 (CI 1.07-2.17)[46]. However, again recent evidence has found the benefit of chromoendoscopy over HD-WLI to be marginal in this setting, with a 2021 meta-analysis of three RCTs not reaching statistical significance (OR 1.17, CI 1.81-1.70)[47-49].

Irrespective, widespread uptake of chromoendoscopy has been limited by the increase in procedure time required for dye application. A 2019 meta-analysis in IBD surveillance found the total procedure time to be a mean of 21.69 min (CI 9.01-34.38) longer for chromoendoscopy[50]. One method to counter this was described by Repici *et al*[51], using oral dye (methylene blue) ingested at the time of bowel preparation. Promisingly, this led to an 8.5% increase in ADR without increasing procedure times, although there was no difference in detection of larger or more advanced polyps.

Virtual chromoendoscopy

Virtual, or electronic chromoendoscopy have been developed in attempt to digitally recreate the enhanced mucosal visualisation of chromoendoscopy without increasing procedure time. However, no

form of virtual chromoendoscopy has been able to conclusively demonstrate a benefit with respect to polyp detection at colonoscopy.

Narrow-band imaging

Narrow-band imaging (NBI) uses optical filters to produce two narrow bands of light centred at wavelengths of 415 nm and 540 nm, corresponding to the primary and secondary light absorption peaks of haemoglobin. Superficial capillaries appear brown, highlighted by the 415 nm wavelength, while deeper vessels in the mucosa and submucosa are cyan due to the deeper penetration of the 540 nm wavelength[52].

The role of NBI in adenoma detection during routine colonoscopy in the general population has been extensively studied. Studies that have found a benefit for NBI in this setting have demonstrated an improvement particularly in the detection of flat or depressed lesions (Figure 1), with a pooled RR of 1.96 in a 2012 meta-analysis[53-56]. However, the majority of studies, including a 2012 Cochrane review by Nagorni *et al*[57], have shown no difference in overall adenoma detection[32,57-61]. In fact, one 2017 RCT demonstrated a reduction in ADR with NBI when adjusted for increased withdrawal time[62].

Multiple possible factors may contribute to the limitations of NBI in screening colonoscopy. Earlier-generation NBI resulted in a reduction in overall brightness due to the narrow bandwidths, which may limit overall visualisation in the wide colorectal lumen. The second-generation bright NBI has been developed to counter this, although recent studies have again demonstrated no difference in overall adenoma detection[58,63]. NBI also appears to be disproportionately affected by poor bowel preparation (which may also be in part due to reduced brightness), with a 2019 meta-analysis finding superior adenoma detection with second-generation NBI only in patients with maximal bowel preparation scores[64]. In addition, the colour spectrum of NBI is different to WLI and therefore may require experience and familiarity with the technology in order to be effective. This was demonstrated by Minamide *et al*[63] who retrospectively reviewed 1831 patients that underwent colonoscopy using second-generation bright NBI or WLI and found a higher polyp detection rate (PDR) with NBI (80.9% *vs* 71.4%, $P = 0.02$) in academic centres familiar with its use, while in community centres, there was actually a trend towards a higher PDR with WLI (51.1% *vs* 47.7%). Additionally, in the NBI group, the ADR for NBI-experienced proceduralists was 63.2% *vs* 39.2% for NBI-inexperienced proceduralists ($P < 0.001$).

i-SCAN

i-SCAN is a software-based post-processing technology, which digitally enhances WLI output through surface and contrast enhancement (i-SCAN mode 1) as well as tone enhancement (i-SCAN modes 2 and 3)[52]. Evidence has again been inconsistent regarding its efficacy for adenoma detection. Multiple studies have found an improvement in polyp and adenoma detection, the largest of which demonstrated a non-statistically significant improvement in ADR from 27% to 33% ($P = 0.33$)[65-68]. As demonstrated by Kidambi *et al*[69] in 2019, this effect has mainly been due to improved detection of diminutive, flat, right-sided adenomas[68,69]. In terms of high-risk populations, Bisschops *et al*[70] found a reduction in adenoma miss rates from 62% to 12% using i-SCAN in 61 patients with HNPCC. On the contrary, a 2012 prospective back-to-back study comparing HD-WLI with i-SCAN modes 1 and 2 in 389 screening colonoscopies showed no difference in ADR or adenoma miss rates, while a 2014 meta-analysis also demonstrated no difference in ADR[71,72]. There is therefore insufficient evidence to recommend routine use of i-SCAN in screening colonoscopy at this stage.

Flexible spectral imaging colour enhancement

Flexible spectral imaging colour enhancement (FICE) also involves digital enhancement of WLI images from the video processor, emphasising certain wavelengths which can be determined by the proceduralists according to 10 factory-determined pre-set modes[52]. FICE was developed with the goal of providing mucosal enhancement without compromising the familiarity of colour patterns from WLI. While one early back-to-back colonoscopy study in 2012 demonstrated reduced adenoma miss rate using FICE[73], multiple studies have demonstrated no significant impact, with the largest RCT in 2010 by Aminalai *et al*[74] finding no difference in ADR between FICE and HD-WLI over 1318 colonoscopies.

Linked colour imaging

Linked colour imaging (LCI) uses both pre- and post-processing technology with narrow wavelength light to separate colours, increasing the vividity of the red and white colour spectrums and enhancing the contrast of mucosal surface patterns and superficial capillaries (Figure 2). It was developed with the aim of enhancing lesion visibility and surface characterisation without compromising brightness or familiarity of colour spectrums, offering perhaps the most promising early evidence for improved adenoma detection[75-77]. It has been demonstrated to improve lesion visibility in both video- and image-based studies when compared to HD-WLI, particularly for nongranular, flat lesions[75,78,79]. While evidence varies with regard to overall ADR, studies have found improvements in proximal adenoma detection and miss rates[80-84]. In addition, a 2020 meta-analysis of 7 studies including 3097 patients demonstrated improved adenoma detection (RR 1.26, $P < 0.001$), particularly in the right colon

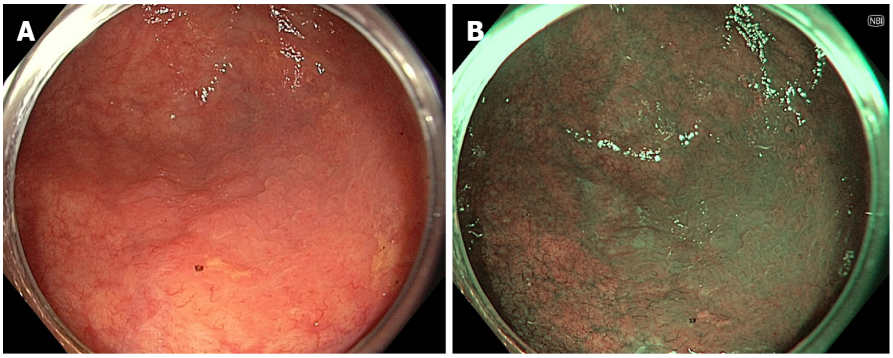

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Figure 1 Sessile serrated adenoma/polyp seen on high-definition white light imaging and narrow-band imaging. A: High-definition white light imaging; B: Narrow-band imaging.

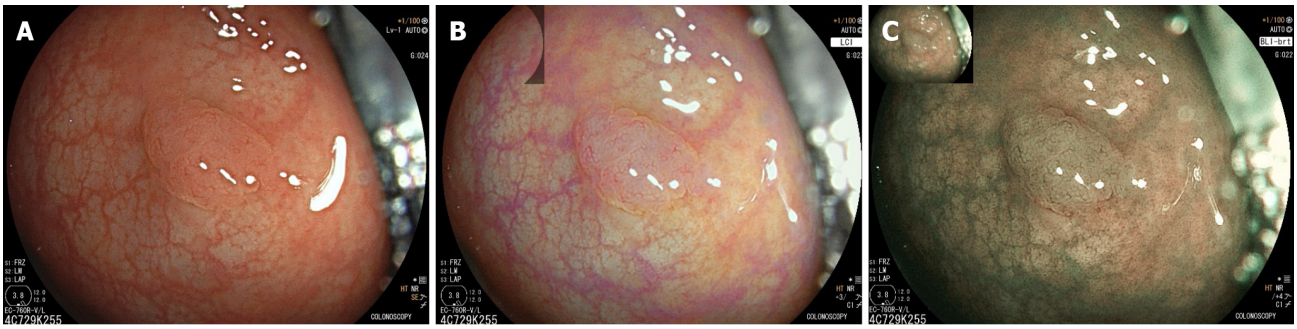

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Figure 2 Sessile serrated adenoma seen on white light imaging, linked colour imaging, and blue light imaging. A: White light imaging; B: Linked colour imaging; C: Blue light imaging.

(RR 2.68, $P < 0.001$) and a mean of 0.27 additional adenomas detected per colonoscopy[85]. In a high-risk population of patients with HNPCC, LCI was found to improve ADR compared to HD-WLI (36.3% *vs* 25.6%, $P = 0.04$)[86]. Interestingly, while advanced imaging such as NBI appears to have a greater impact when used by experienced endoscopists, a 2021 study by Hasegawa *et al*[87] found a strong negative correlation between the baseline ADR with HD-WLI and the improvement ratio, indicating that perhaps the familiar colour pattern allows effective use by non-expert proceduralists.

Blue light imaging

Blue light imaging (BLI) is form of digitally enhanced imaging which concentrates and enhances a specific wavelength of light between 410-450 nm, increasing the contrast of superficial micro-vessels and mucosal surface structures (Figure 2). BLI uses four independent light-emitting diodes rather than the xenon light used in NBI, which is postulated to improve brightness[88]. This new technology has not been as extensively studied, however a video-based 2015 study demonstrated improved visibility scores with BLI bright mode compared to WLI according to both expert and non-expert proceduralists[89]. On a smaller scale this translated into improved adenoma detection, with two studies (including 182 and 127 patients respectively), finding an improvement in ADR from 27.8% to 46.2% ($P = 0.01$) and a reduction in adenoma miss rate from 10% to 1.6% ($P = 0.001$) compared to HD-WLI[90,91]. In contrast, the largest prospective study to date, including 963 patients, did not find a difference in ADR, though did find a non-statistically significant increase in mean adenomas per patient (APP) (1.27 *vs* 1.01, $P = 0.08$)[92].

Texture and colour enhancement imaging

Texture and colour enhancement imaging (TXI) is a recently developed technology, where the HD-WLI image is split into two layers, each individually undergoing brightness enhancement, tone mapping and texture enhancement before the images are stacked (TXI mode 1) and undergo further colour enhancement (TXI mode 2)[93]. Similarly to LCI, this aims to enhance mucosal visualisation without compromising familiarity of colour patterns or brightness (Figure 3). As an only recently developed technology, clinical studies examining adenoma detection are not yet available, however preliminary studies have demonstrated improved visibility of adenomas and sessile serrated polyps using TXI compared to HD-WLI[94,95].

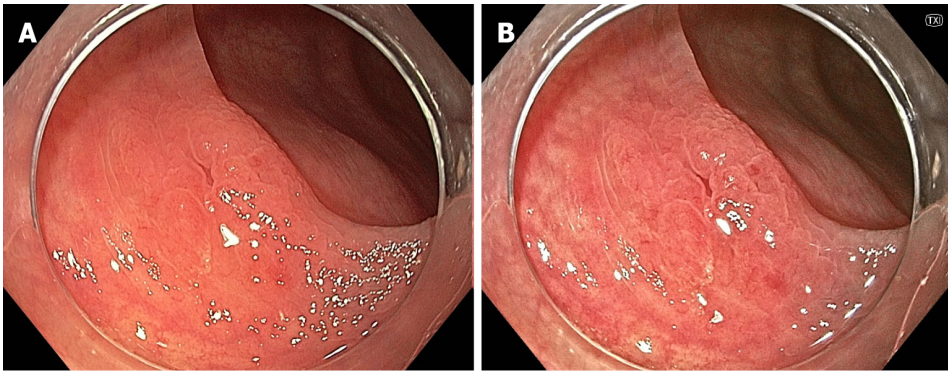

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Figure 3 Sessile serrated adenoma seen on white light imaging and texture and colour enhancement imaging. A: White light imaging; B: Texture and colour enhancement imaging.

Virtual chromoendoscopy summary

While virtual chromoendoscopy theoretically offers enhanced mucosa visualisation without the increase in procedure time required for dye-based chromoendoscopy, none of the currently available technologies have conclusively demonstrated a meaningful improvement in ADR compared to HD-WLI. These technologies may all have a role particularly in improving detection of flat, right sided adenomas and may be used as additional tools for examination during screening colonoscopy, but evidence is not yet sufficient for recommendation in societal guidelines. Data appear most promising for newer forms of post-processing technology where brightness and familiarity of color patterns are preserved, however additional research is required to confirm this efficacy.

Autofluorescence imaging

Light of a specific wavelength induces cell autofluorescence produced by endogenous fluorophores, with varied characteristics between normal (green), inflamed (dark green) and neoplastic (magenta) tissue. Autofluorescence imaging (AFI) relies on the detection and delineation of this natural fluorescence after stimulating the mucosal cells with short wavelength light[96,97]. In doing so, AFI aims to detect neoplastic or dysplastic tissue even before it manifests as an anatomically distinguishable discrete lesion. McCallum *et al*[98] demonstrated that colonic adenomas have a significantly higher autofluorescence intensity than non-neoplastic polyps. It is therefore unsurprising that the greatest impact of AFI across multiple studies has been improved detection of flat, right sided polyps rather than elevated polypoid adenomas, with one RCT reporting an ADR for flat neoplasms of 42.5% *vs* 29.2% ($P < 0.001$)[99,100]. However, a 2015 meta-analysis found that while the adenoma and polyp miss rates were lower with AFI, there was no difference in overall ADR despite an average of 8 min longer procedural time for the AFI group[101].

Artificial intelligence

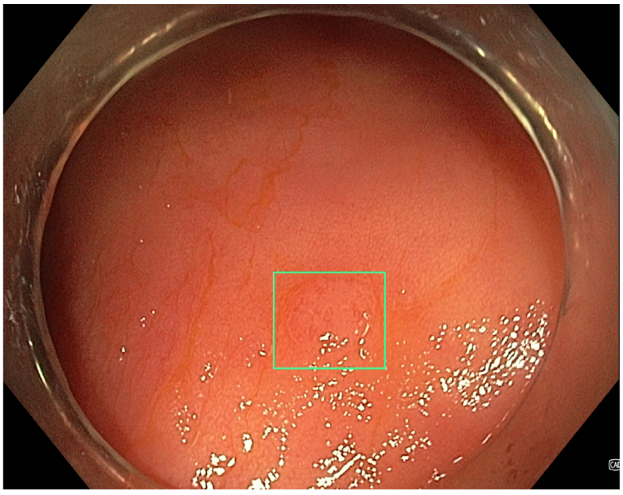
Multiple AI systems have been developed for polyp detection during surveillance colonoscopy, referred to as computer aided detection (CADe). These systems use convulational neural networks (CNNs) which are trained using still images and videos of polyps[102]. The most recent systems then output a real-time alert to the proceduralist to the presence of the polyp, most commonly with a square around the perimeters of the image output or around the polyp itself (Figure 4). CADe systems were initially analysed in still image- and video-based studies, demonstrating a sensitivity of 95%-99% and accuracy of 96%[103-107]. Subsequently, large studies by Repici *et al*[102] (2020) and Wang *et al*[108,109] (2019 and 2020) in real-time AI-assisted colonoscopy have demonstrated an increase in ADR (RR 1.61 *vs* 1.30), as well as a 1.46- to 1.72-fold increase in total adenomas detected. The adenoma miss rate in tandem colonoscopy studies has also been demonstrated to be lower with CADe-assisted colonoscopy (14%-20%) compared to WLI (31%-40%)[110,111]. Subsequently, multiple meta-analyses have consistently demonstrated improved ADR and adenomas detected per colonoscopy with CADe systems (Table 1)[112-115].

An alternative role for AI-assistance in screening colonoscopy is based on quality assurance, employing AI to monitor withdrawal speed, endoscope slipping and blind spots to ensure consistency in colonoscopic practice. Gong *et al*[116] studied ENDOANGEL for this purpose and in a 2020 RCT involving 704 patients demonstrated an odds ratio of 2.3 for adenoma detection. Similar results were demonstrated by Su *et al*[117] using their Automatic Quality Control System (AQCS). Although not yet explored in studies, it may be that the combination of these AI systems using quality control and CADe may facilitate optimal adenoma detection. This is an area for further study as these systems become more widely available.

Table 1 Meta-analyses on efficacy of real-time computer aided detection

Ref.	Studies, patients	ADR			Adenoma per patient			Withdrawal time	False positives
		AI	WLI	RR	AI	WLI	Mean difference	Mean difference CADe *vs* control	
Aziz *et al*[112], 2020	3 studies, 2815 patients	32.9%	20.8%	1.58	0.47	0.26	0.20	0.9 min (P = 0.03)	4.87% (n = 137)
Hassan *et al*[113], 2021	5 studies, 4354 patients	36.6%	25.2%	1.44	0.58	0.36	0.22	0.34 min (P = 0.13)	-
Spadaccini *et al*[114], 2021	6 studies, 5178 patients	34.0%	26.6%	1.78	-	-	-	No significant difference	-
Barua *et al*[115], 2021	5 studies, 4311 patients	29.6%	19.3%	1.52	0.41	0.23	0.18	0.5 min	11.2%

ADR: Adenoma detection rate; AI: Artificial intelligence; WLI: White light imaging; RR: Relative risk; CADe: Computer aided detection.



DOI: 10.3748/wjg.v28.i47.6632 **Copyright** ©The Author(s) 2022.

Figure 4 Computer aided detection detection system with a real-time alert seen around a flat tubular adenoma.

Fluorescence molecular endomicroscopy

Fluorescence molecular endomicroscopy (FME) involves targeted fluorescent agents that bind to specific cellular components of dysplastic cells, allowing detection using a specialised near infra-red FME (NIR-FME) probe[118]. For example, Hartmans *et al*[119] developed a fluorescently-labelled antibody against vascular endothelial growth factor A (which is upregulated in colonic adenomas) and injected this intravenously 3 d prior to colonoscopy. In their pilot study, all 39 adenomas from 15 patients were detected using the NIR-FME probe, demonstrating the feasibility of this technique[119]. Alternatively, Joshi *et al*[120] identified a peptide sequence that binds specifically to sessile serrated adenomas/polyps (SSA/Ps) which was administered topically using a spray catheter to 38 subjects undergoing routine outpatient colonoscopy, distinguishing SSA/Ps from normal colonic mucosa with 89% sensitivity and 92% specificity[120].

Problem: Over surveillance?

As a result of the expanding range of advanced imaging technologies (Table 2) and improved adenoma detection, patients will increasingly meet societal guidelines for more frequent surveillance colonoscopy. To counter this, guidelines may eventually need to be adjusted to reduce the frequency of colonoscopy based on diminishing adenoma miss-rates. However, a 2014 study by Gómez *et al*[121] demonstrated no difference in adenoma detection at follow-up colonoscopy after prior procedures completed by higher ADR endoscopists using HD-WLI. Currently, the duration of use of these advanced technologies has been insufficient to analyse polyp detection at future surveillance, hence further research is required as experience grows.

Table 2 Summary of strengths and weakness of advanced imaging technologies in adenoma detection

Modality		Strengths	Weaknesses
HD-WLI		Widely available	Marginal incremental benefit over SD-WLI
		Increased detection of flat, right-sided adenomas and SSAs	
Chromoendoscopy		Increased detection of small and flat adenomas	No significant increase in detection of advanced adenomas
		Increased dysplasia detection in IBD (compared to SD-WLI)	Increased procedural time
		May increase polyp detection in high-risk syndromes (serrated polyposis syndrome, HNPCC)	
Virtual chromoendoscopy	NBI	May improve flat lesion detection	Loss of brightness and familiarity of colour patterns
		Effective in those with experience using NBI	No evidence of increased total adenoma detection
			Less effective when used by proceduralists inexperienced in NBI
	i-SCAN	May reduce miss-rates in high-risk populations	Not widely available
			No difference in adenoma detection in larger studies
			Insufficient evidence to recommend use
	FICE	Retains familiar colour patterns	Not widely available
			No difference in ADR
	LCI	Retains familiar colour patterns	Not widely available
		Effective when used by non-LCI experienced proceduralists	Variable evidence regarding overall adenoma detection
		Improve adenoma detection, particularly right sided and flat lesions	
	BLI	Improved adenoma detection and miss rate in smaller studies	Not widely available
			No difference in ADR in largest study to date
	TXI	Retains familiar colour patterns	Not widely available
			New technology therefore insufficient evidence
AFI		Improved detection of flat/right sided polyps	Not widely available
			Increased procedure time
			No difference in overall ADR
AI		Improves ADR	Expensive currently
		Improves consistency between proceduralists	Not widely available
		Quality assurance	Some increase in procedure time
FME		In theory may improve detection of flat/poorly visible polyps	Insufficient evidence
			Requires injection/ingestion of tracer

HD-WLI: High-definition white light imaging; SD-WLI: Standard definition white light imaging; HNPCC: Hereditary non-polyposis colon cancer; NBI: Narrow-band imaging; IBD: Inflammatory bowel disease; BLI: Blue light imaging; TXI: Texture and colour enhancement imaging; FME: Fluorescence molecular endomicroscopy; AI: Artificial intelligence; AFI: Autofluorescence imaging; SSA/Ps: Sessile serrated adenomas/polyps; ADR: Adenoma detection rate.

POLYP CHARACTERISATION

Importance of polyp characterisation

Polyp characterisation is critically important for both small and larger polyps. In the context of diminutive (< 5 mm) and small (< 10 mm) polyps, accurate characterisation has facilitated the "resect and discard" and "do not resect" strategies. For larger polyps, accurate endoscopic characterisation guides the selection of suitable polyps for endoscopic resection as well as the most appropriate resection technique.

Diminutive and small polyps

Traditionally, all polyps identified during colonoscopy have been resected and examined histologically. However, as the accuracy of endoscopic identification of polyps has improved, the "resect and discard" or even "do not resect" strategies have been developed to minimise the resource consumption of routine histological analysis. These strategies were developed after large studies found that advanced histology (at least high-grade dysplasia) is present in as few as 1.7% of diminutive (≤ 5 mm) polyps, and only 6.6%-10.0% of small (< 10 mm) polyps[122,123]. In fact, a 2013 meta-analysis including 6280 polyps found only 56.7% of diminutive polyps are even neoplastic[124]. On this basis, the American Society of Gastrointestinal Endoscopy (ASGE) published the Preservation and Incorporation of Valuable endoscopic Innovations (PIVI) thresholds for adopting real-time endoscopic assessment of polyps for "resect and discard" and "do not resect"[125]. For diminutive polyps to be discarded without pathological assessment, endoscopic imaging should provide a ≥ 90% agreement in assignment of post-polypectomy surveillance. Polyps > 5 mm in size should be sent for histological assessment given the up to 10% frequency of more advanced histology which would alter surveillance intervals[123]. For diminutive rectosigmoid hyperplastic polyps, imaging should provide ≥ 90% negative predictive value for adenomatous histology. Even hyperplastic-appearing diminutive polyps proximal to the sigmoid colon should be resected as these polyps have a more than 10% chance of being SSA/Ps histologically [126]. These strategies would result in significant cost-savings to the healthcare sector. For example, Solon *et al*[127] examined the potential financial impact of this strategy for the National Health Service (NHS) in England in 2016, demonstrating potential annual cost savings of £141192057.

Larger polyps

For larger polyps, endoscopic characterisation is critical to guiding suitability for endoscopic resection as well as appropriate resection techniques. Even for non-interventionalists who are not proceeding with immediate resection, accurate characterisation without the need for biopsy may be ideal to guide appropriate referral. Kuroha *et al*[128] highlighted this in a 2021 study examining predictors of success in 369 colorectal ESDs. Severe fibrosis was associated with increased mean procedure time, as well as lower en bloc and complete resection rates, with the greatest predictors of severe fibrosis on multivariate analysis being prior resection attempt (OR 175.4) and pre-treatment biopsy (OR 8.3)[128]. In addition, pre-resection biopsies can be inaccurate in large lesions, with false negative rates as high as 86% for adenocarcinoma, therefore characterisation with advanced imaging and upfront endoscopic resection may be more appropriate[129].

Training in polyp characterisation

Accurate polyp characterisation using advanced mucosal imaging is impacted to some extent by proceduralist experience. A 2014 video-based study demonstrated that interventional endoscopists specialising in complex polypectomy were more accurate in identifying malignant polyps when compared to other endoscopists[130]. However, multiple studies support the efficacy of specialised training in advanced mucosal imaging for polyp characterisation, irrespective of endoscopist experience. Both Bae *et al*[131] and Patel *et al*[132] have studied the accuracy of endoscopists before and after a training module on identification diminutive rectal polyps, in whom the negative predictive value (NPV) for diminutive neoplastic polyps improved from 82.1% to 92.5%-94.7%, thus meeting the PIVI threshold. In addition, studies have demonstrated accurate characterisation after training even in medical residents with no endoscopy experience, while Basford *et al*[133] found no difference in the accuracy of interpretation of HD-WLI and i-SCAN images prior to specific training between consultant gastroenterologists, trainees and medical students[133-136]. Proceduralists should therefore engage in specific training in advanced mucosal imaging rather than relying on experience alone, in order to improve accuracy of polyp characterisation.

Classifications systems

Multiple polyp classification systems have been developed to improve polyp characterisation (Table 3). While not reliant on advanced imaging, the Paris classification aids in risk stratification for larger polyps prior to consideration of endoscopic resection (Figure 5)[137]. A large multicentre 2017 study found that the presence of any 0-IIc ("depressed") component predicted submucosal invasive cancer in almost 30% of patients. In laterally spreading tumours (LSTs), the presence of an elevated component (0-IIa + Is) predicted submucosal invasion in over 10% over patients *vs* 4.9% for those with flat lesions alone (0-IIa)

Table 3 Summary of existing classification systems using advanced mucosal imaging

System	Imaging modality	Polyp features	Accuracy	Complexity	TA/TVAs included	SSAs included
Kudo	Any	Pits	AUC 0.94[143]	Complex	Yes	No
NICE	NBI	Vessels and pits	Sensitivity 98%, NPV 97.8%[145]	Moderate	Yes	No
JNET	NBI	Vessels and pits	AUC 0.97 for JNET 1, 0.84 for JNET 2A, 0.9 for JNET 3 but less accurate for JNET 2B (AUC 0.72)[152]	Moderate	Yes	No
BASIC	BLI	Vessels, pits and surface	Accurate surveillance prediction in 90%, NPV for rectosigmoid polyps 91%[160]	Moderate	Yes	No
WASP	Any	Pits, surface, shape	May improve SSA detection[162]	Simple	No	Yes
mSano	NBI	Vessels, pits and surface	AUC 0.92[169]	Simple	Yes	Yes

NBI: Narrow-band imaging; WASP: Workgroup serrAted polypS & polyposis; TVA: Tubulo-villous adenomas; AUC: Area under the receiver operating characteristic curve; NPV: Negative predictive value; BASIC: Blue light imaging adenomas serrated international classification; JNET: Japan NBI Expert Team developed the Japan NBI expert team; NICE: NBI International Colorectal Endoscopic; LCI: Linked colour imaging; SSA: Sessile serrated adenomas.

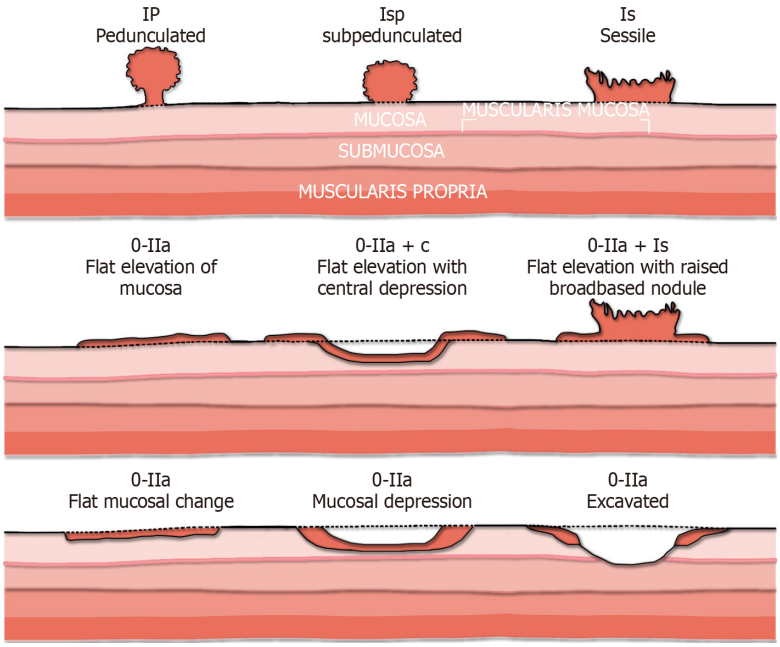


Figure 5 Paris classification. Citation: Mathews AA, Draganov PV, Yang D. Endoscopic management of colorectal polyps: From benign to malignant polyps. *World J Gastrointest Endosc* 2021; 13: 356-370[141].

(P < 0.001)[138]. However there is considerable inter-observer variability, particularly with regard to classification of lesions as flat *vs* sessile, with one study finding a kappa statistic of 0.42[139]. Van Doorn *et al*[139] proposed a simplified classification system of "pedunculated", "elevated" (including flat and sessile) and "depressed" in order to address this, which resulted in improved interobserver agreement and 91.6% accuracy for prediction of invasive cancer[140].

Kudo classification

The Kudo classification (Figure 6) was developed in 1996 to classify polyps according to their "pit patterns" on magnifying endoscopy[141]. Type I pits appear round, while type II appear stellate or papillary, both representing benign changes (normal, hyperplastic or inflammatory). Type III-s pits are smaller, round, tubular pits while type III-L are larger tubular pits, representing tubular adenomas (TA). Type IV pits are branch-like or gyrus-like and represent tubulo-villous adenomas (TVA), while type V pits are non-structured representing HGD or cancer[142]. Multiple studies have assessed the accuracy of the Kudo classification, summarised by a 2014 meta-analysis of 20 studies, including 5,111 colorectal

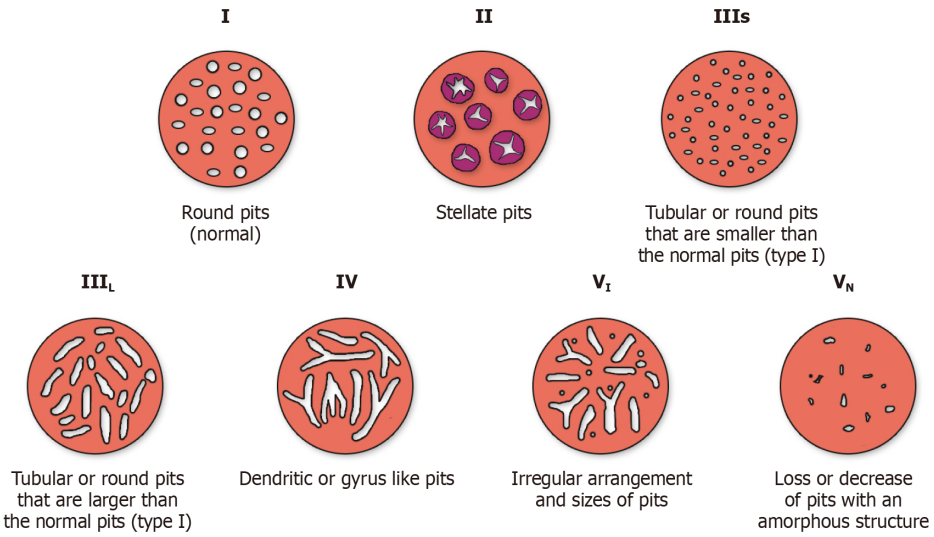


Figure 6 Kudo's classification. Citation: Mathews AA, Draganov PV, Yang D. Endoscopic management of colorectal polyps: From benign to malignant polyps. *World J Gastrointest Endosc* 2021; 13: 356-370[141].

lesions[143]. Pit pattern classification differentiated neoplastic from non-neoplastic polyps with a pooled sensitivity of 89.00%, specificity of 85.78% and area under the receiver operating characteristic curve (AUC) of 0.94[144].

NBI International Colorectal Endoscopic classification

The NBI International Colorectal Endoscopic (NICE) classification was developed in 2012 with the goal of developing an international consensus for classification using NBI[145]. This classification takes into account the polyp colour, vessel pattern and surface pattern to characterise polyps into NICE type 1 (hyperplastic) type 2 (adenoma) and type 3 (invasive cancer) (Figure 7). Using this simplified classification results in highly accurate differentiation of neoplastic from non-neoplastic polyps, with sensitivity of 97%-99%, specificity of 85%-95% and accuracy of 89%-98% across 3 Large prospective studies[145-147]. In the 2012 validation study, 471 predominantly diminutive and small polyps were predicted with high-confidence with sensitivity of 98% and NPV of 97.7%, while 119 low-confidence predictions resulted in a sensitivity of 94.2% and NPV of 94.4%, both easily exceeding PIVI thresholds [145]. However, in a study of 2123 larger lesions, the NICE classification predicted deep invasive cancer with a sensitivity of just 58.4%. Nevertheless, due to low rates of deep invasion this was still associated with an NPV of 96.4% and specificity of 98.1%, therefore the authors suggested that even large NICE 1 and 2 Lesions should be considered for endoscopic resection[148]. The NICE classification has also been validated in a smaller cohort using i-SCAN rather than NBI, with similar results[149].

Japan NBI expert team classification

More recently, the Japan NBI Expert Team developed the Japan NBI expert team (JNET) classification specifically for the classification of colorectal polyps based on their appearance on magnification NBI using a combination of vessel and surface pattern analysis (Figure 8)[150,151]. The JNET classification is highly accurate for differentiating neoplastic *vs* non-neoplastic polyps, with an AUC of 0.97 for JNET 1 (hyperplastic/SSA/Ps) and 0.84 for JNET 2A (adenoma with LGD) in a 2020 meta-analysis[152]. In a retrospective 2020 study, this resulted in an increase in the number of adenomas resected per colonoscopy (1.7 *vs* 1.2, $P < 0.01$) and a reduction in resection of non-neoplastic lesions (8.9% *vs* 17.0%, $P < 0.01$)[153]. It is also highly specific in predicting deep invasive cancer in JNET 3 Lesions, with specificity of 100% and an AUC of 0.9[152]. In addition, unlike other systems, the JNET classification has been validated for characterisation of dysplasia within SSA/Ps, with Murakami *et al*[154] finding that the presence of JNET 2A/B/3 foci within a JNET 1 Lesion is 83.9% sensitive, 95.5% specific and 94.5% accuracy for detection of dysplasia within sessile serrated lesions. However, the main limitation of the JNET classification is in the interpretation of JNET 2B lesions, with studies demonstrating a wide range of advanced pathology, from HGD to superficial invasive cancer and even deep invasive cancer in JNET 2B polyps, with an AUC of 0.72[152,155,156]. This was highlighted in a recent study that retrospectively reviewed 297 colorectal adenocarcinomas, in which the probability of deep invasion was only 1.8% for JNET 2A, 30.1% for JNET 2B and 96.6% for JNET 3[157,158]. In this study, JNET 2B lesions were then further analysed using chromoendoscopy and Kudo"s classification of pit patterns. In Kudo non-V lesions, the risk of deep invasion was only 4.3%. Overall, JNET differentiates accurately for JNET 1, 2A, and 3 lesions, however proceduralists should consider further examination with magnified chromoendoscopy for JNET 2B lesions to improve accuracy of histology prediction.

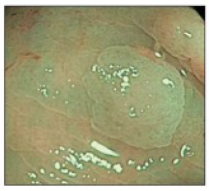
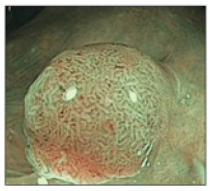
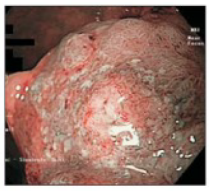
	Type 1	Type 2	Type 3
Color	Same or lighter than background	Browner relative to background (verify that color arises from vessels)	Brown to dark brown relative to background, sometimes patchy whiter areas
Vessels	None or isolated lacy vessels coursing across the lesion	Brown vessels surrounding white structures	Has area(s) with markedly distorted or missing vessels
Surface pattern	Dark or white spots of uniform size, or homogeneous absence of pattern	Oval, tubular, or branched white structures surrounded by brown vessels	Areas with distortion or absence of pattern
Most likely pathology	Hyperplastic	Adenoma	Deep submucosally invasive cancer
			

Figure 7 Narrow-band Imaging International Colorectal Endoscopic classification. Citation: Puig I, Kaltenbach T. Optical Diagnosis for Colorectal Polyps: A Useful Technique Now or in the Future? *Gut Liver* 2018; 12: 385-392[150]. Copyright© The Author(s) 2018. Published by The Korean Society of Gastroenterology, the Korean Society of Gastrointestinal Endoscopy, the Korean Society of Neurogastroenterology and Motility, Korean College of Helicobacter and Upper Gastrointestinal Research, Korean Association the Study of Intestinal Diseases, the Korean Association for the Study of the Liver, Korean Pancreatobiliary Association, and Korean Society of Gastrointestinal Cancer (Supplementary material).

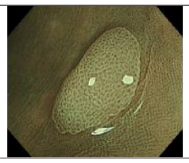
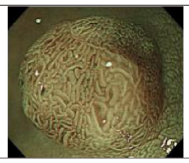
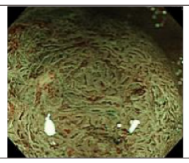
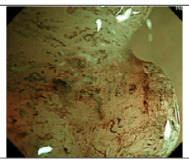
	Type 1	Type 2A	Type 2B	Type 3
Vessel pattern	•Invisible	•Regular caliber •Regular distribution (meshed/spiral pattern)	•Variable caliber •Irregular distribution	•Loose vessel areas •Interruption of thick vessels
Surface pattern	•Regular dark or white spots •Similar to surrounding normal mucosa	•Regular (tubular/branched/papillary)	•Irregular or obscure	•Amorphous areas
Most likely histology	Hyperplastic polyp/ Sessile serrated polyp	Low grade intramucosal neoplasia	High grade intramucosal neoplasia/ Shallow submucosal invasive cancer	Deep submucosal invasive cancer
Endoscopic image				

Figure 8 Japan Narrow-band Imaging Expert Team developed the Japan Narrow-band Imaging expert team classification. Citation: Hirata D, Kashida H, Iwatate M, Tochio T, Teramoto A, Sano Y, Kudo M. Effective use of the Japan Narrow Band Imaging Expert Team classification based on diagnostic performance and confidence level. *World J Clin Cases* 2019; 7: 2658-2665[158]. Copyright© The Author(s) 2019. Published by Baishideng Publishing Group Inc (Supplementary material).

BLI adenomas serrated international classification

The BLI adenomas serrated international classification (BASIC) classification was developed in 2018 for classification of polyps using BLI, based on assessment of surface, pit patterns and vessels, classifying polyps as either hyperplastic, traditional adenomatous, sessile serrated or cancer[159]. In the largest prospective validation study of 748 diminutive polyps this classification reached PIVI thresholds with accurate surveillance prediction in 90% and an NPV for rectosigmoid polyps of 91%[160].

Dutch Workgroup serrAted polypS & polyposis classification

The dutch workgroup serrAted polypS & polyposis (WASP) classification was developed in 2016 to facilitate accurate differentiation of SSA/Ps from hyperplastic and traditional adenomatous polyps as many existing classification systems did not allow for inclusion of SSA/Ps[161]. It's accuracy has been validated by Lee *et al*[162] who demonstrated that the implementation of a specific training program in the WASP classification led to a statistically significant increase in SSA/P resection over the 6-mo

training period, from 4.5% to 8% (P = 0.003).

Sano and mSano classification

The Sano classification (Figure 9) characterises polyps according to their capillary pattern, with barely visible honeycomb pattern capillaries in type I (normal or hyperplastic), larger elongated capillaries in type II [adenoma with low-grade dysplasia (LGD)] and irregular branching vessels in type III [high-grade dysplasia (HGD)] or adenocarcinoma)[163,164]. In a validation study, 97% of Sano II lesions were diagnosed as LGD while 87% of Sano III lesions were HGD or invasive cancer[165]. In 2013, this system was modified by Singh *et al*[166] (mSano classification) to include type IIo lesions in order to distinguish hyperplastic from sessile serrated polyps (Figure 7). Across multiple studies, the overall accuracy of the mSano classification has been between 90%-97%, with near-perfect interobserver agreement (k 0.89)[166,167]. The NPV for diminutive rectosigmoid polyps is as high as 100% and the accuracy for post-polypectomy surveillance 97%, exceeding the PIVI thresholds described above[167]. mSano as a standalone classification system was compared to the combination of the WASP and JNET classification in 2020, with superior high-confidence predictions (85% *vs* 69%, P < 0.05) and equivalent interobserver reliability[168]. It was also compared to the NICE classification in a 2018 RCT including 348 colonoscopies, with an AUC of 0.92 for prediction of neoplasia by mSano *vs* 0.78 for NICE (P = 0.02) and an AUC of 0.92 for prediction of suitability for endoscopic resection *vs* 0.83 for NICE (P = 0.04)[169]. The mSano is therefore a highly accurate standalone criteria for characterisation of colonic polyps including differentiation of neoplasia (including SSA/Ps) as well as invasive cancer.

HD-WLI

There appears to be some incremental benefit from examination with HD-WLI alone *vs* SD-WLI for polyp characterisation, although this may be smaller than expected. In the largest direct comparison from Rastogi *et al*[31] in 2011, HD-WLI improved sensitivity for characterisation of small adenomas from 51.7% to 66.8% (P < 0.001) however the overall accuracy did not change. Minimal evidence exists comparing the accuracy of HD-WLI to SD-WLI for characterisation and prediction of invasion in larger polyps, however with the vast expansion of advanced imaging technologies, evidence increasingly supports the use of ancillary technology over HD-WLI in this context[170].

Chromoendoscopy

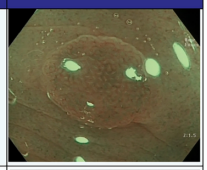
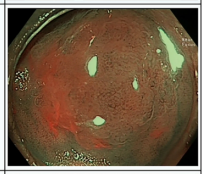
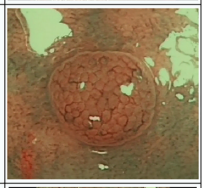
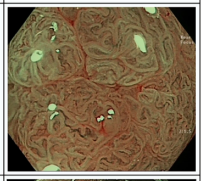
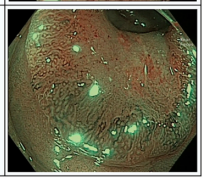
Chromoendoscopy has been demonstrated to be highly effective in differentiating neoplastic from non-neoplastic small colonic polyps, with overall diagnostic accuracy of greater than 99%[171-173]. However, with increasingly accurate forms of virtual chromoendoscopy for assessment of these diminutive and small polyps, the procedure time required for chromoendoscopy is likely to limit its ongoing use. Instead, the main ongoing role for chromoendoscopy may be in the prediction of invasion depth in larger lesions to guide resection techniques[174]. For example, the European Society of Gastrointestinal Endoscopy (ESGE) guidelines recommend the use of chromoendoscopy for pit pattern analysis in JNET 2B lesions (where NBI lacks accuracy), in order to further qualify the risk of deep invasion according to Kudo's classification as described above[175]. This recommendation has been supported by Hosotani *et al*[157] in their 2021 study which demonstrated a PPV of 76% for invasive cancer in the presence of a "VH" pit pattern and a NPV of 96% for non-V pit patterns. Even in this context however, a recent prospective study including 400 patients found that there was no overall incremental benefit for the use of chromoendoscopy in addition to HD-WLI and NBI for the characterisation of large nonpedunculated polyps[176]. Novel indications for chromoendoscopy include the use of acetic acid chromoendoscopy or submucosal methylene blue injection (Figure 10) to clearly delineate polyp margins prior to resection[177-179].

Virtual chromoendoscopy

While virtual chromoendoscopy has not been conclusively demonstrated to improve polyp detection, an expanding body of evidence supports its use for polyp characterisation to guide endoscopic resection strategies, as well as the "resect and discard" and "do not resect" strategies in diminutive polyps. For classification of highly prevalent small and diminutive polyps where dye-based chromoendoscopy may no longer be efficient on a population level, virtual chromoendoscopy has been demonstrated to have equivalent accuracy with a reduction in median procedural and interpretation time[180].

NBI

For diminutive colorectal polyps, multiple studies have shown that characterisation using NBI is able to easily exceed PIVI thresholds, with correct surveillance interval prediction in 92%-99% of cases and an NPV for diminutive rectosigmoid polyps of 91%-92%[62,124,181,182]. While many of these studies have been performed by expert endoscopists with experience in NBI, it has also been demonstrated that non-interventional endoscopists are able to achieve significant improvement following specific training, with Higashi *et al*[133] reporting an overall accuracy of 90% for non-interventionalists following a single training module[133,183].

MS classification (predicted histology)	Description	Example
MS I (HP - hyperplastic polyp)	Pale colour ± round pits with central brown star-like dots or bland appearance ± minute capillaries that may meander across polyp	
MS IIo (SSA/P - sessile serrated adenoma/polyp)	Pale or light dark colour ± open pits ± 3 out of 5: cloud-like surface, inconspicuous margins, mucous cap, irregular shape and varicose microvascular vessels	
MS II (tubular adenoma with low grade dysplasia)	Light dark or dark colour ± white linear or oval pits ± linear or oval regular capillary network surrounding pits	
MS IIIa (high grade dysplasia‡/ villous or tubulovillous adenoma/superficial cancer)	Light dark or dark colour ± white villous/cerebriform pits ± tortuous/branched mildly regular capillary network surrounding pits§	
MS IIIb (invasive cancer)	Dark surroundings with pale central area ± loss of pit and vascular pattern	

‡High-grade dysplastic SSA/Ps are included in this category
§May have slight loss of pit pattern and vascularity when leaning towards superficial cancer

Figure 9 mSano classification demonstrating delineation between sessile serrated adenomas/polyps and hyperplastic polyps. Citation: Zorron Cheng Tao Pu L, Yamamura T, Nakamura M, Koay DSC, Ovenden A, Edwards S, Burt AD, Hirooka Y, Fujishiro M, Singh R. Comparison of different virtual chromoendoscopy classification systems for the characterization of colorectal lesions. *JGH Open* 2020; 4: 818-826[168]. Copyright© The Author(s) 2019. Published by Journal of Gastroenterology and Hepatology Foundation and John Wiley & Sons Australia, Ltd (Supplementary material).

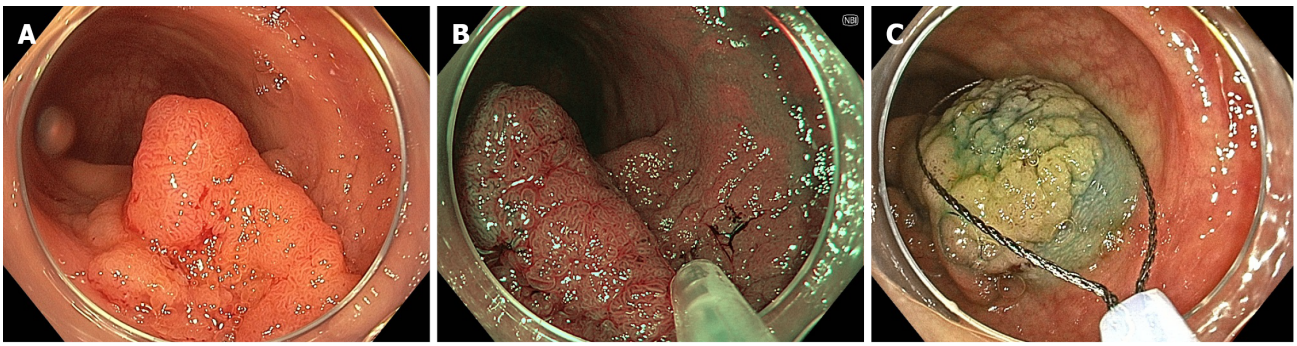


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Figure 10 Large colonic laterally spreading tumour. A: White light imaging with poor differentiation between polyp and normal tissue; B: Flat extension seen more clearly on NBI; C: Submucosal methylene blue injection prior to resection clearly delineating the margins of the flat spreading component.

Additionally, NBI has been used for the characterisation and prediction of invasion depth within larger colonic polyps (Figure 11). As early as 2008, Katagiri *et al*[165] demonstrated that an irregular capillary pattern (designated CP III) on NBI predicted a 65.6% (21/31) rate of invasive adenocarcinoma. Subsequently, Ikematsu *et al*[184] differentiated CP III into IIIA (characterised by high microvessel density with a lack of uniformity, blind ending, branching and curtailed irregularly) and IIIB (characterised by the presence of a nearly avascular or loose microvascular area). They found that IIIA lesions defined adenomas, intramucosal cancers and superficial submucosal invasive cancer, while IIIB lesions defined deep submucosal invasive cancers, with a sensitivity of 84.8%, specificity of 88.7% and overall accuracy of 87.7%. NBI has also been examined for detection of dysplasia and cancer within SSA/Ps. Tate *et al*[185] found that the presence of an adenomatous (NICE II) pattern within an SSA had 95%

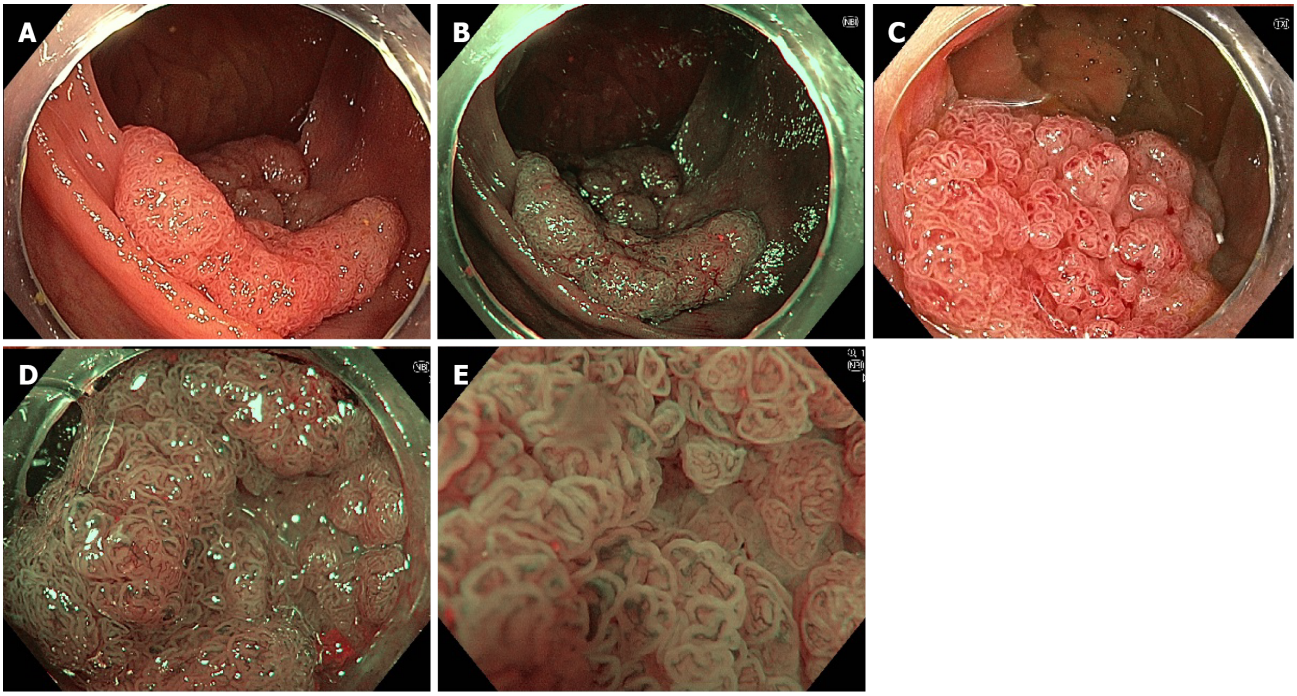

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Figure 11 Laterally spreading tubulo-villous adenoma with high-grade dysplasia. A: White light imaging; B: Narrow-band imaging (NBI); C: Texture and colour enhancement imaging; D: NBI with magnification; E: NBI with high-magnification using the underwater technique.

accuracy and a 98.1% NPV for detection of dysplasia within SSA/Ps, while Chino *et al*[186] demonstrated 100% sensitivity and 99% specificity with NBI for detection of cancers within SSA/Ps.

FICE

FICE has also been demonstrated to be highly accurate for the characterisation of colorectal polyps, with sensitivity of 89.4%-94.7%, specificity of 81.0%-89.2% and accuracy of 87.0%-89.4%[172,187-190]. However, Yoshida *et al*[187] did demonstrate its accuracy to be inferior to that of chromoendoscopy (89.4% *vs* 94.7%, $P < 0.05$). While minimal direct comparative data exists between modalities of virtual chromoendoscopy, Akarsu *et al*[191] found the NPV of FICE (80%) to be inferior to that of NBI (96.3%, $P < 0.001$), although there was no difference in overall accuracy.

i-SCAN

i-SCAN has achieved similar results with respect to diminutive and small colorectal polyp categorisation, with sensitivity and specificity consistently above 90% across multiple studies[149,192-194]. It also appears to be an accessible form of advanced imaging for non-experts, with junior residents achieving similar accuracy to experts in one study after a 30-min training session[149]. There have been two RCTs directly comparing the accuracy of NBI and i-SCAN for polyp characterisation, both of which have found no difference in accuracy between these modalities but did demonstrate superiority for both NBI and i-SCAN when compared to HD-WLI[195,196].

LCI

LCI was developed in conjunction with BLI, aiming to improve polyp detection while BLI aimed to improve characterisation (Figure 12). Accordingly, minimal evidence exists regarding the accuracy of LCI for polyp characterisation. However, in 2017 Wu *et al*[197] employed the NICE classification using LCI, and reported a sensitivity of 96.5%, specificity of 83.8% and NPV of 93.9% for neoplastic lesion prediction.

BLI

The accuracy of BLI for polyp characterisation has been more extensively studied. Both retrospective and prospective studies have demonstrated the superiority of BLI over WLI for the characterisation of < 10 mm colonic polyps, with the largest 2019 prospective randomised study by Rondonotti *et al*[198] finding the overall accuracy for BLI to be 92% *vs* 84% for WLI ($P = 0.01$)[198-200]. BLI has also been compared to NBI using the JNET classification in a retrospective study where there was no significant difference in accuracy (92.1% for BLI *vs* 91.7% for NBI)[201].

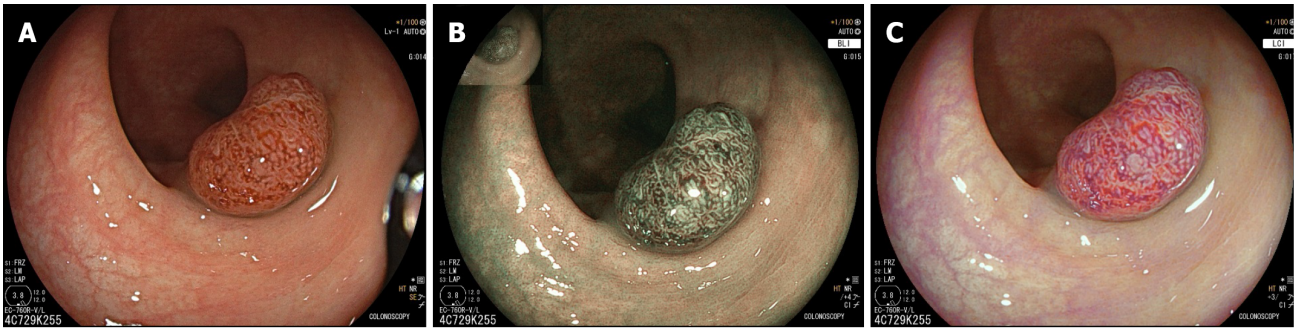

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Figure 12 Tubulo-villous adenoma. A: White light imaging; B: Blue light imaging; C: Linked colour imaging.

AFI

While studies on AFI have been promising regarding polyp detection, its role in polyp characterisation appears limited. A 2011 RCT comparing HD-WLI, AFI and NBI did find that the overall accuracy of AFI is equivocal to that of NBI for distinguishing adenoma from hyperplastic polyps (84.9% *vs* 88.4%)[202]. However, the interobserver agreement for NBI with magnification is superior to that of AFI, while a 2017 meta-analysis demonstrated inferior specificity using AFI (44%) compared to NBI (69%, $P = 0.031$) [203,204].

TXI

As the most recently developed form of advanced mucosal imaging, TXI has yet to be studied in the context of polyp characterisation. Given the familiarity of color patterns, it may have some role for differentiation of neoplastic from non-neoplastic diminutive polyps which may increase its uptake during population screening. In addition, this familiarity may benefit proceduralists during resection by more clearly delineating polyp margins without compromising visualisation.

Artificial intelligence

Extensive research has been undertaken in recent times into the development of AI systems for characterisation of colonic polyps, designated computer aided diagnosis (CADx) (Table 4). These systems have proven to be highly accurate in assessment of diminutive polyps, with a 2020 meta-analysis demonstrating a pooled AUC of 0.96 (CI 0.95-0.98) and a pooled NPV of 95.1%[104]. Interestingly, across multiple studies, CADx systems have not proven to be superior to expert endoscopists regarding histology prediction, although they have consistently led to improved histology prediction in non-expert endoscopists, nearing that of experts[205-208]. In these studies, the NPV for diminutive polyps has been 90%-97%, with an accurate surveillance interval in 93-94%, well surpassing PIVI thresholds [205,206,208-220].

There are fewer studies examining the efficacy of AI for delineation of submucosal invasive adenocarcinoma to guide resection strategies. Lu *et al*[221] found the accuracy of their AI model "Endo-CRC" to be 93.78% for polyps with and 91.71% for polyps without advanced CRC. Lui *et al*[222] developed an AI model to classify polyps more than 2 cm in size as being endoscopically resectable (less than 1 mm submucosal invasion, no lymphovascular invasion and no more than well-differentiated adenocarcinoma) or non-resectable. The overall accuracy was 85.5% for prediction of endoscopically resectable lesions, but improved to 94.3% when the AI system was interpreting NBI images. However, while AI models have been more effective than non-expert interventionalists for detection of invasive carcinoma, in each of these studies AI was not superior to expert endoscopists, suggesting the main role of AI for larger polyps may be in improving inter-endoscopist consistency as well as perhaps aiding in selection of suitable referrals to interventionalists by non-expert endoscopists[221,222].

In vivo histologic diagnosis

Emerging technologies have been developed with the goal of achieving *in vivo* histological diagnosis, termed "optical biopsy". Accurate optical biopsies would allow endoscopists to not only surpass PIVI thresholds for small and diminutive polyps but would also allow accurate endoscopic diagnosis for larger polyps and LSTs where existing mucosal imaging technology may have deficiencies.

Endocytoscopy

Endocytoscopy is a novel technology that allows *in vivo* visualisation of tissue at the cellular level in real-time[223]. The device can either be incorporated into the endoscope or comes as a probe-based system, utilising a high-power fixed-focus objective lens to achieve ultra-high magnification in excess of 450 ×, generally following methylene blue staining[224]. Studies have demonstrated superior accuracy compared to advanced mucosal imaging and chromoendoscopy, with accuracy as high as 93.3%-96.8%

Table 4 Studies on the accuracy of AI for polyp histology prediction

Ref.	Study type	Imaging modality	Number of patients/polyps	Sensitivity	Specificity	NPV	Accurate surveillance interval
Kominami *et al* [209], 2016	Retrospective	NBI	41 patients, 118 polyps	93%	95%	93%	92.7%
Chen *et al*[210], 2018	Retrospective	NBI	284 polyps	96%	78%	90%	-
Mori *et al*[211], 2018	Prospective	NBI	325 patients, 466 polyps	93%	90%	95%	-
Renner *et al*[212], 2018	Retrospective	WLI, NBI	100 polyps	92%	63%	90%	-
Byrne *et al*[213], 2019	Retrospective	NBI	125 polyps	98%	83%	97%	-
Min *et al*[214], 2019	Prospective	LCI	91 patients, 217 polyps	83%	70%	71%	-
Sánchez-Montes *et al*[206], 2019	Retrospective	WLI	225 polyps	92%	89%	87%	-
Horiuchi *et al*[215], 2019	Prospective	AFI	95 patients, 258 polyps	80%	95%	93%	-
Ozawa *et al*[216], 2020	Retrospective	WLI, NBI	309 polyps	97% for NBI, 90% for WLI	-	91% for NBI, 85% for WLI	-
Jin *et al*[205], 2020	Retrospective	NBI	300 polyps	83%	90%	94%	-
Zacharia *et al*[208], 2020	Retrospective	WLI, NBI	524 polyps	96%	90%	93%	94%
Rodriguez-Diaz *et al*[217], 2021	Retrospective	NBI	119 patients, 280 polyps	96%	84%	91%	94%
Van der Zander *et al*[218], 2021	Retrospective	WLI, BLI	54 patients, 60 polyps	96%	93%	88%	-
Yoshida *et al*[220], 2021	Retrospective	BLI	25 patients, 100 polyps	91%	85%	92%	-
Sakamoto *et al* [219], 2022	Retrospective	WLI, BLI	604 polyps	96% for WLI, 96% for BLI	84% for WLI, 89% for BLI	-	-

NBI: Narrow-band imaging; NPV: Negative predictive value; BLI: Blue light imaging; WLI: White light imaging; LCI: Linked colour imaging.

for distinction of neoplastic *vs* non-neoplastic diminutive polyps[225]. Endocytoscopy has been shown to be similarly highly accurate for larger polyps in detection of submucosal invasion, with an overall accuracy of 85.8%-97.0%[226-229]. The main limiting factors for this technology are the requirement for specific equipment, as well as the time and training required to facilitate accurate interpretation of the images. However, its uptake may evolve with the development of AI technologies which could allow effective use by inexperienced proceduralists. Misawa *et al*[230] developed and published a new AI system for interpretation of endocytoscopy images (using NBI rather than methylene blue staining) named "EndoBRAIN" in 2016. Their study demonstrated overall sensitivity, specificity, and accuracy for high-confidence predictions of 97.6%, 95.8%, and 96.9% respectively. In 2020, Kudo *et al*[231] compared "EndoBRAIN" to trainee and expert endoscopists using both dye-based and virtual chromoendoscopy and found the AI system to be superior to both groups, with sensitivity of 96.9%, specificity of 100% and overall accuracy of 98%.

Multiphoton microscopy

Multiphoton microscopy is based on the detection of signals at specific emission wavelengths after laser excitation, offering real-time high-resolution visualisation. The use of longer photons allows deeper tissue penetration and visualisation up to a depth of several hundred microns[232]. Recently, Terradillos *et al*[233] developed an AI system for interpretation of multiphoton microscopy images of colorectal polyps, with a specificity of 91% and sensitivity of 82% for malignant colorectal lesions. Further study is clearly required into the application of this technology, however the greater depth of visualisation may allow *in vivo* assessment of invasion depth for submucosal invasive adenocarcinoma.

CONCLUSION

New and existing advanced mucosal imaging technologies facilitate improved adenoma detection and characterisation in both expert and non-expert endoscopists (Table 5). The use of virtual chromoendoscopy for polyp detection has been limited by reduced brightness and loss of familiarity of color patterns, however new technologies such as LCI and TXI enhance visualisation without significantly altering color patterns and may lead to more consistent improvement in polyp detection. Additionally, the availability of AI systems is increasing and may improve consistency between expert and non-expert endoscopists. Advanced mucosal imaging also allows accurate *in vivo* assessment of polyps to guide resection techniques, while clearly exceeding PIVI thresholds for the "resect and discard" and "do not resect" strategies. NBI has been at the forefront of polyp characterisation, improving delineation of neoplastic from non-neoplastic diminutive and small polyps, while improving prediction of invasion depth in larger polyps. AI technologies are yet to surpass expert endoscopists for histology prediction but facilitate accurate prediction by non-experts to rival that of expert endoscopists. Effective use of these advanced mucosal imaging technologies is not out of reach of any endoscopist following brief but dedicated training programs, thereby maximising the efficacy of everyday colonoscopy and improving patient outcomes.

Table 5 Summary and conclusions for each form of advanced mucosal imaging discussed

Modality		Detection	Characterisation	Comment
HD-WLI		Advantages: Marginal benefit in overall adenoma detection; and improved detection of right-sided, flat polyps, and SSAs[1]	Advantage: Marginal benefit for small adenomas; disadvantage: Insufficient evidence for large polyps	Advantage: Widely available
Chromo-endoscopy		Advantage: Increases polyp detection; disadvantage: Increases withdrawal time	Advantages: Highly effective for small polyps (although inefficient); and useful in prediction of invasion depth for large polyps[1]	Disadvantage: Increases procedural time
Virtual chromo-endoscopy	NBI	Disadvantage: No significant difference in ADR	Advantages: Accurate for distinguishing neoplastic from non-neoplastic small and diminutive polyps; and accurate for prediction of invasion depth[1]	Disadvantage: Loss of brightness; neutral: Greater efficacy when used by expert proceduralists
	i-SCAN	Neutral: Variable results, increased detection of flat and right-sided polyps	Advantage: Effective for diminutive and small polyps	
	FICE	Disadvantage: No significant difference in ADR	Disadvantage: Inferior to NBI	Advantage: Familiar colour spectrum
	LCI	Advantages: Improves adenoma detection; and effective for non-expert proceduralists[1]	Disadvantage: Insufficient evidence	Advantage: Familiar colour spectrum
	BLI	Disadvantage: No significant difference in ADR	Advantage: Similar to NBI in terms of colour spectrum and accuracy[1]	Advantage: Similar colour spectrum to NBI
	TXI	Advantage: Increases polyp visibility in image-based studies	Disadvantage: Insufficient evidence	Disadvantage: Insufficient evidence; advantage: Familiar colour spectrum
AFI		Disadvantage: Insufficient evidence; advantage: Improves detection of flat, right-sided polyps and reduces miss rates	Disadvantage: Inferior to NBI	Disadvantage: Not widely available
AI		Advantage: Increases adenoma detection; no significant difference in withdrawal time[1]	Advantages: Highly accurate; superior to non-expert endoscopists for histology prediction; not superior to experts using NBI[1]	Disadvantage: Not yet widely available
FME		Disadvantages: Expensive; insufficient evidence		Disadvantage: Not widely available
Endo-cystoscopy			Neutral: Accurate but requires expertise for interpretation; advantages: Uptake may increase with incorporation of AI	Disadvantages: Requires additional equipment; and not widely available
Multiphoton microscopy			Disadvantage: Insufficient evidence	Disadvantage: Requires additional equipment

[1]The most promising technologies.

HD-WLI: High-definition white light imaging; NBI: Narrow-band imaging; BLI: Blue light imaging; TXI: Texture and colour enhancement imaging; FME:

Fluorescence molecular endomicroscopy; AFI: Autofluorescence imaging; ADR: Adenoma detection rate; AI: Artificial intelligence; LCI: Linked colour imaging; SSA: Sessile serrated adenomas.

FOOTNOTES

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REVIEW

Acute liver injury in COVID-19 patients hospitalized in the intensive care unit: Narrative review

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Abstract

In recent years, humanity has been confronted with a global pandemic due to coronavirus disease 2019 (COVID-19), which has caused an unprecedented health and economic crisis worldwide. Apart from the respiratory symptoms, which are considered the principal manifestations of COVID-19, it has been recognized that COVID-19 constitutes a systemic inflammatory process affecting multiple organ systems. Across the spectrum of organ involvement in COVID-19, acute liver injury (ALI) has been gradually gaining increasing attention by the international scientific community. COVID-19 associated liver impairment can affect a considerable proportion of COVID-19 patients and seems to correlate with the severity of the disease course. Indeed, COVID-19 patients hospitalized in the intensive care unit (ICU) run a greater risk of developing ALI due to the severity of their clinical condition and in the context of multi-organ failure. The putative pathophysiological mechanisms of COVID-19 induced ALI in ICU patients remain poorly understood and appear to be multifactorial in nature. Several theories have been proposed to explain the occurrence of ALI in the ICU setting, such as hypoperfusion and ischemia due to hemodynamic instability, passive liver congestion as a result of congestive heart failure, ischemia-reperfusion injury, hypoxia due to respiratory failure, mechanical ventilation itself, sepsis and septic shock, cytokine storm, endotheliitis with concomitant coagulopathy, drug-induced liver injury, parenteral nutrition and direct cytopathic viral effect. It should be noted that no specific therapy for COVID-19 induced ALI exists. Therefore, the therapeutic approach lies in preventive measures and is exclusively

supportive once ALI ensues. The aim of the current review is to scrutinize the existing evidence on COVID-19 associated ALI in ICU patients, explore its clinical implications, shed light on the underlying pathophysiological mechanisms and propose potential therapeutic approaches. Ongoing research on the particular scientific field will further elucidate the pathophysiology behind ALI and address unresolved issues, in the hope of mitigating the tremendous health consequences imposed by COVID-19 on ICU patients.

Key Words: Liver injury; COVID-19; Intensive care; Pathophysiological mechanisms; Cytokine storm; Multi-organ failure

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Core Tip: In recent times, coronavirus disease 2019 (COVID-19) pandemic has substantially altered the hitherto existing medical landscape, causing tremendous perturbations among the global scientific community and imposing a disproportionate burden on healthcare systems worldwide. It soon became apparent that COVID-19 affects multiple organ systems, including the liver. Acute liver injury has been progressively identified as a common, yet often under-recognized, complication of COVID-19, especially in the intensive care unit (ICU) setting, resulting in higher mortality rates. This review attempts to elucidate the underlying pathophysiological mechanisms that contribute to the development of acute liver injury in ICU patients with COVID-19, summarize emergent data and propose therapeutic strategies.

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INTRODUCTION

In recent years, humanity has been confronted with a global pandemic due to coronavirus disease 2019 (COVID-19), which has caused an unprecedented health and economic crisis worldwide. The initial isolation of the novel severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) and the subsequent first outbreak of COVID-19 in Wuhan, China, marked a grim milestone for mankind, substantially altering the existing scientific, social and economic landscape[1-3]. Since then, COVID-19 has rapidly spread throughout the world across all nations and ages and has resulted in the emergence of numerous mutant SARS-CoV-2 variants, triggering recurrent COVID-19 surges that continue to drive the ongoing pandemic[4-6]. Due to its dynamic and ever-changing epidemiological course, COVID-19 still remains an international public health emergency and accounts for considerable morbidity and mortality, inflicting an overwhelming death toll and a disproportionate burden on healthcare systems worldwide, thus raising major concerns among the global scientific community[7,8].

The clinical spectrum of COVID-19 is highly variable with a wide range of clinical manifestations. In most cases, the course of the disease is either asymptomatic or presents as a mild self-limited infection; however, in some patients, especially those with underlying comorbidities, COVID-19 evolves to a severe or even a critical life-threatening disease, culminating in the development of acute respiratory distress syndrome (ARDS) which requires intensive care support and may eventually progress to death [9,10]. Undoubtedly, SARS-CoV-2 infection demonstrates an inherent propensity for the respiratory system; hence, lung involvement is the predominant feature in patients hospitalized with COVID-19. Nonetheless, as the complete magnitude of COVID-19 sequelae continues to unravel, it has been realized that this new nosological entity constitutes a diverse, complex and multifaceted syndrome that extends beyond the respiratory system and affects multiple organs, including the liver[11,12]. Bearing this in mind, specific attention has been gradually drawn towards the pathogenesis of liver injury in the setting of COVID-19, since acute liver injury (ALI) has been progressively identified as a common, yet often under-recognized, complication of COVID-19. As a matter of fact, there have been numerous literature reports that a considerable proportion of COVID-19 patients develop liver injury characterized by abnormalities in liver chemistry levels. Notably, ALI seems to correlate with the severity of the disease course[13-16]. Indeed, COVID-19 patients hospitalized in the intensive care unit (ICU) run a greater risk of developing ALI, mainly due to the severity of their clinical condition and in the context of multi-organ failure, thus resulting in higher mortality rates[17-20].

Studies dealing with COVID-19 induced ALI in the ICU setting are rather limited. Accordingly, the present review aims to summarize relevant existing evidence regarding ALI in COVID-19 patients

hospitalized in the ICU and explore its clinical implications, while at the same time an attempt is made to elucidate the underlying pathophysiological mechanisms that contribute to the development of ALI in ICU patients, and to propose therapeutic approaches.

DEFINING ACUTE LIVER INJURY

A universal definition of ALI is still lacking. This poses significant challenges and oftentimes generates confusion among clinicians when referring to liver injury and trying to accurately interpret abnormal liver tests[21]. Based on the latest American College of Gastroenterology clinical guidelines for the evaluation of abnormal liver chemistries[22], markers of liver injury comprise routinely measured liver chemistries, namely aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP) and bilirubin, whereas markers of hepatocellular function include albumin, bilirubin and prothrombin time. Sometimes, elevation of gamma-glutamyl transferase (GGT) can be used as a supplement to confirm the hepatic origin of an elevated ALP level. According to the same guidelines [22], AST and/or ALT elevation is defined as borderline when values are less than 2-fold the upper limit of normal (ULN); mild when they are between 2 and 5 times the ULN; moderate when they are between 5 and 15 times the ULN; severe if they exceed 15 times the ULN; and massive in case they are above 10000 IU/L. Furthermore, in order to characterize the type of liver injury, the following categories have been proposed based on the proportion of elevated AST and ALT values as compared to ALP values: Hepatocellular injury (disproportionate elevation of AST/ALT), cholestatic injury (predominant elevation of ALP) and mixed pattern of injury (elevation of both AST/ALT and ALP levels). For this purpose, the R ratio has been used, which is derived from the formula R = (ALT value:ALT ULN)/(ALP value:ALP ULN), whereby an R ratio > 5 indicates hepatocellular injury, an R value < 2 suggests cholestatic injury and an R value 2-5 is consistent with a mixed pattern of liver injury[22].

The lack of a uniform definition of ALI has led to the arbitrary use of different criteria for identifying ALI, which has resulted in great heterogeneity of reported research results. The adoption of diverse criteria by researchers, either for defining ALI or characterizing its pattern and severity, is also evident in the current literature regarding COVID-19 induced ALI. For instance, in a large retrospective United States cohort of COVID-19 patients, ALI was defined as any elevation of ALT and AST above normal and was subsequently classified, according to the degree of ALT elevation, as none/mild, moderate or severe when ALT values were less than 2-fold the ULN, between 2 and 5 times the ULN and more than 5-fold the ULN respectively[23]. On the other hand, Yip *et al*[24] defined ALI as an elevation of ALT and/or AST ≥ 2 times the ULN, with a concomitant increase of total bilirubin by more than 2-fold the ULN and/or international normalized ratio (INR) ≥ 1.7[24], whereas Cai *et al*[25] defined liver injury either as an increase of ALT and/or AST ≥ 3 times the ULN or as a more than 2-fold increase of ALP, GGT and/or total bilirubin above the ULN[25]. Meanwhile, other researchers avoided the use of the term ALI and employed other general terminology instead, such as ''liver test abnormalities'' or ''abnormal liver function tests'', to include any increase of at least one of AST, ALT, ALP, bilirubin and GGT above the ULN (as *per* local laboratory reference range standards) or even a reduction of albumin levels below normal cut-off values[26,27].

Based on the above, it is more than obvious that there is a wide discrepancy in the current COVID-19 literature with regard to ALI definition, thus rendering interpretation and comparison of results rather burdensome and obscure. On this account, it would be desirable for researchers to reach a consensus on a unifying definition in order to eliminate existing disparities.

EXISTING EVIDENCE

In the current COVID-19 literature there is a dearth of published clinical studies with an exclusive focus on COVID-19 induced ALI in ICU patients. Moreover, most of the available data on the topic are rather diverse owing to the lack of a uniform definition for liver injury, disparate thresholds applied for ALI, divergent study designs and different endpoints of each study.

The allegedly first study to explore the incidence, clinical characteristics and outcomes of ALI exclusively in ICU patients with COVID-19 was conducted in Germany and included 72 critically ill patients between March and July 2020[28]. The investigators used the term severe liver dysfunction and defined it as the occurrence of hypoxic liver injury (manifested by elevated aminotransferase levels > 20-fold the ULN in the setting of cardiac, circulatory or respiratory failure and after exclusion of other possible causes) and/or jaundice (total bilirubin ≥ 2 mg/dL). They found that 31% of the ICU patients developed severe liver dysfunction during their ICU stay approximately a week after ICU admission, predominantly in the form of cholestatic liver injury (45%) followed by equally contributing hepatocellular (27%) and mixed pattern injury (27%). Patients with severe liver dysfunction had a higher simplified acute physiology score II on admission, indicating a more severe clinical condition, as well as higher rates of viremia during their ICU stay. Severe liver dysfunction was associated with more severe respiratory failure, as manifested by more frequent development of ARDS and lower values of

Horowitz index, namely lower ratio of partial pressure of oxygen to fraction of inspired oxygen (PaO_2/FiO_2 ratio). Furthermore, patients with severe liver dysfunction were more likely to require mechanical ventilation (MV), circulatory support with vasopressors, renal replacement therapy and rescue therapy by means of veno-venous extracorporeal membrane oxygenation (ECMO). Accordingly, patients with severe liver dysfunction experienced higher mortality rates and, more importantly, severe liver dysfunction was found to be an independent predictor of mortality[28].

Along the same lines, Huang *et al*[29] conducted a retrospective study with the aim to investigate the prevalence of hypoxic hepatitis in 51 COVID-19 patients hospitalized in a Chinese ICU from December 2019 to March 2020[29]. They used the same definition of hypoxic hepatitis; that is, a massive but transient elevation of ALT levels > 20-fold the ULN in the setting of cardiac, circulatory or respiratory failure, after excluding other putative causes of ALI. Based on this terminology, hypoxic hepatitis was evident in 3 male patients, corresponding to 5.88% of all ICU patients, with peak ALT values being 1665, 1412 and 1140 U/L respectively. All 3 patients progressed to respiratory failure and eventually died due to multiple organ failure. When looking at the dynamic changes of liver enzymes, it was observed that ALT and AST elevations occurred at an earlier stage, while bilirubin levels increased sharply at a later stage when transaminase values had already started to show a downward trend after peaking in the third week. The authors came to the conclusion that hypoxic hepatitis was not an infrequent condition in ICU patients with severe COVID-19 and was accompanied by high mortality[29].

Meanwhile, Salik *et al*[30] conducted a study which included 533 COVID-19 patients admitted to the ICU with the aim to determine the impact of observed liver test abnormalities on mortality[30]. Liver injury was defined as ALT and/or AST levels above 3 times the ULN, and/or total bilirubin levels greater than 2-fold the ULN. Patients were divided into three groups: Group 1 consisted of patients with normal liver chemistries; Group 2 included patients with abnormal liver test values not falling into the category of liver injury; Group 3 comprised patients with liver injury. Any kind of liver test abnormality, reflected by groups 2 and 3, was observed in 52% of the total cohort and was more frequent in males. However, as *per* study's definition, only 8.6% of all ICU patients developed ALI, represented by group 3 alone. Paradoxically, the authors reported that patients with ALI and milder liver test abnormalities (groups 2 and 3) had shorter ICU lengths of stay compared with group 1, but this result is most likely attributed to the unacceptably high total mortality rates of patients belonging to these groups (71.4% and 78.3% for groups 2 and 3 respectively), which erroneously were not taken into account as confounding factors during the analysis. The authors concluded that liver test abnormalities were predictive of higher mortality, given that groups 2 and 3 experienced higher than expected 7-d, 28-d and total mortality rates[30].

A similar definition of ALI was employed by Arentz *et al*[31] who published the first case series of ICU patients with COVID-19 in the United States, dating back to February-March 2020[31]. A total of 21 patients were included. On ICU admission, abnormal liver tests were reported in 8 patients (38%) with one patient displaying very high levels of AST (4432 U/L) and ALT (1414 U/L). During the course of the disease, among patients who were mechanically ventilated, 3 (14.3%) developed ALI, defined as a more than 3-fold rise above the ULN in AST or ALT levels. No further details about ALI were provided by this study, since its focus was not on liver injury in the ICU, but rather its aim was to describe the clinical characteristics and outcomes in the initial ICU cases of COVID-19 patients in the United States [31].

Likewise, in a retrospective observational cohort study from France with 600 COVID-19 patients, 153 of whom required ICU hospitalization, it was reported that 9.8% of the ICU patients developed ALI, defined as AST levels higher than 3-fold the ULN[32]. ALI occurred more frequently in the ICU compared to common ward patients, as did most of the other extrapulmonary complications, like acute kidney injury, cardiovascular events and thromboembolic events. Data analysis demonstrated that factors associated with higher risk for ALI included age > 75 years, concomitant cancer, chronic cardiac disease, as well as higher levels of C-reactive protein (CRP), serum creatinine and hemoglobin[32].

Another retrospective observational study from China reported an 18.1% incidence of ALI in 83 ICU patients with COVID-19[33]. In this case, ALI was defined as an elevation of hepatic biomarkers more than 2-fold the ULN or as disproportionately elevated AST and ALT levels compared to ALP levels. However, the focus of this study was on acute gastrointestinal injury (defined as various grades of malfunction of the gastrointestinal tract) in critically ill patients with COVID-19 and not specifically on ALI; hence, no additional data on ALI were available. Nevertheless, since ALI could be considered one of the components of acute gastrointestinal injury, it could be assumed that the main study conclusions may also be applicable to ALI. The major findings of this study were that patients with worse grades of acute gastrointestinal injury had worse clinical severity features, while sequential organ failure assessment score, MV duration and white blood cell count arose as independent risk factors for the development of more severe acute gastrointestinal injury. What is more, these patients exhibited higher rates of both septic shock and 28-d in-hospital mortality[33].

In a case series examining the temporal evolution of blood liver tests in 20 consecutive ICU patients with COVID-19, liver injury was reported to be frequent, albeit transient and non-severe[34]. However, liver injury was ill-defined as any elevation of at least one liver test (AST, ALT, ALP, GGT, total bilirubin) above the ULN. Consequently, all 20 patients exhibited some form of liver injury during the first 10 d after ICU admission. Interestingly, only median levels of AST, ALT and GGT (but not bilirubin

or ALP) increased above the ULN, while only GGT showed pronounced elevations (≥ 3-fold the ULN) with a peak on day 8 after ICU admission. Based on these findings, the researchers commented that late cholestasis was frequently observed[34].

In addition, Shousha *et al*[35] conducted a prospective cohort study in 547 Egyptian patients with COVID-19 in order to investigate the underlying prevalence and severity of liver and gastrointestinal disturbances, as well as their effect on disease outcomes[35]. Among patients who required ICU admission (122/547), 48.50% had elevated AST and 35.60% had elevated ALT. Elevated AST, but not ALT, levels were associated with increased mortality in univariate analysis. Moreover, patients admitted to the ICU displayed significantly higher hospital admission levels of fibrosis-4 index (FIB-4), which incorporates 4 variables (age, AST, ALT and platelet count) and is considered a predictive marker for significant liver fibrosis. Similarly high levels of FIB-4 were observed in severe COVID-19 cases and in non-survivors. In the multivariate analysis, FIB-4 score > 3.25 was a significant predictor of mortality. Unfortunately, no other specific ICU details could be extrapolated from this study, owing to the trial design and aim, which was not focused on ICU patients[35].

Based on the observation that black population in the United States had been disproportionately affected by COVID-19, Currier *et al*[36] investigated potential differences in outcomes between black and non-black patients with COVID-19 and elevated liver enzymes[36]. They included a total number of 8028 patients, out of whom 3937 patients had available liver test data for interpretation. The analysis demonstrated that 45% of both black and non-black patients exhibited elevations in their liver chemistries. Among black patients with liver test elevations, 46% were intubated compared to 34.8% of non-black patients with elevated liver enzymes. This study highlighted that black patients who had liver test abnormalities were more prone to ICU admission and intubation than non-black counterparts, who albeit run a significantly higher risk of death[36].

Besides, ALI has also been appreciated in the context of specific patient categories with a prior history of liver disease. In this perspective, a multicenter observational cohort study from the United States aimed to explore the prevalence and impact of COVID-19 induced ALI in liver transplant recipients of various races and ethnicities[37]. 112 adult liver transplant recipients with COVID-19 were included, 81 of whom required hospitalization. Among hospitalized patients, 30 (37%) were admitted to the ICU with the majority of them requiring MV and circulatory support with vasopressors. ICU patients were more likely to develop ALI, which was determined according to ALT values at the peak of COVID-19 and was defined as ALT levels greater than 2-fold the ULN. On multivariate analysis, use of vasopressors in the ICU was found to be an independent predictor of liver injury. Furthermore, ICU patients had a higher likelihood of having their immunosuppression therapy modified. However, reduction in immunosuppression was not associated either with ALI or with risk of mortality or graft rejection, in contrast to antibiotic administration which was related to increased risk for ALI. Overall, the presence of ALI independently predicted risk for ICU admission and mortality in liver transplant recipients with COVID-19[37].

Other than cohort studies, there have also been case reports regarding COVID-19 induced ALI in ICU patients. Of note, a case report from Italy highlighted a rare case of ALI progressing to acute liver failure and eventually death[38]. It involved 2 critically ill COVID-19 patients hospitalized in the ICU due to severe ARDS. The patients were treated with tocilizumab, as part of the anti-cytokine storm regimen, which however seemed to have aggravated the underlying COVID-19 immunosuppression, thus facilitating the development of opportunistic infections in the already immunocompromised patients. Indeed, a few days prior to their death, lab tests revealed Herpes simplex virus 1 viremia leading to fulminant hepatitis with dramatic increases in ALT, AST, bilirubin and INR levels and resulting in fatal outcomes for both patients[38].

Furthermore, one should bear in mind that within the spectrum of liver injury in COVID-19 patients lies the cholestatic pattern of liver injury, along with its late sequelae, such as the development of secondary sclerosing cholangitis in critically ill patients. In this regard, Bütikofer *et al*[39] described the incidence and severity of cholestatic liver injury in 34 ICU patients with COVID-19[39]. Cholestatic liver injury was termed mild if ALP and GGT levels were higher than 1.5-fold and 3-fold the ULN respectively, whereas it was considered severe in case the above abnormalities were accompanied by a concomitant elevation of total bilirubin levels more than 2-fold the ULN. The investigators reported that 59% of the ICU patients developed some degree of cholestasis (32% mild and 27% severe). Patients with severe cholestatic injury displayed a more complicated clinical course, required more intensified supportive treatment (in terms of vasopressor support, renal replacement therapy and ECMO) and had a more extended length of ICU stay. Moreover, 4 out of 9 patients with severe cholestatic injury developed secondary sclerosing cholangitis, eventually resulting in 2 deaths and 1 candidacy for liver transplantation. By the same token, irrespective of the degree of cholestasis, the vast majority of patients with pronounced elevations of ALT levels (> 10-fold the ULN) developed untoward outcomes, progressing either to secondary sclerosing cholangitis or death[39].

From a similar perspective, on the grounds of a higher than expected incidence of cholangiopathies in critically ill patients with COVID-19, Wendel-Garcia *et al*[40] addressed the issue of drug-induced liver injury (DILI) in a prospective observational cohort of patients with COVID-19 associated ARDS[40]. They performed a post hoc analysis on 243 ICU patients who were on invasive MV, with the aim to investigate whether a causal relationship between the prolonged infusion of high-dose ketamine and the

occurrence of cholestatic liver injury existed. Acute cholestatic DILI was defined as ALP levels greater than 1.5-fold and GGT levels greater than 3-fold the ULN, whereas patients were deemed to have severe cholestatic liver injury in case a concurrent increase in bilirubin levels more than 2-fold the ULN was present. During their ICU stay, 114 patients developed cholestatic liver injury, 100 of whom had received long-term ketamine infusion, while severe cholestatic liver injury occurred in 33% of the latter. The analysis revealed a duration-effect and dose-effect relationship between ketamine infusion and bilirubin and ALP levels. In other words, prolonged duration of infusion and higher doses of ketamine were positively correlated with rising bilirubin and ALP levels. Interestingly, no such effect was observed with long-term infusion of propofol and sufentanil, even at high doses. The study clearly demonstrated an increased hazard of developing cholestatic liver injury in ICU patients who had received long-term ketamine infusion as a co-sedative agent; yet, no association between ketamine infusion and increased in-hospital mortality was depicted[40].

The aforementioned studies regarding the prevalence and clinical implications of COVID-19 induced ALI in ICU patients are summarized in Table 1.

PATHOPHYSIOLOGY

The pathophysiology of ALI in COVID-19 patients hospitalized in the ICU has not been fully elucidated and still awaits to be unraveled, since the underlying mechanisms have not been sufficiently decoded and appear to be multifactorial in nature. So far, the scientific research conducted in the field has proposed several mechanisms that associate SARS-CoV-2 infection with ALI. These mechanisms may have direct or indirect impact on liver function. In fact, there seems to be a complex interplay among several distinct pathophysiological pathways implicated in the course of critically ill patients with COVID-19 (Figure 1). Presumably, these diverse pathophysiological mechanisms act in synergy and exert cumulative effects, since no single mechanism can completely explain the vast spectrum of liver involvement in ICU patients with COVID-19. It is worth noting that COVID-19 induced ALI can occur either in the context of an underlying liver disease, thus leading to decompensation of the preexisting state of equilibrium, or can manifest as a de novo nosological entity in ''naïve'' patients with no previous history of documented hepatic dysfunction[41-44].

Hypoperfusion

ICU patients represent a specific subgroup of patients with distinctive features. Hemodynamic instability is frequently encountered among patients hospitalized in the ICU setting, who therefore require administration of vasopressors or even inotropes, sometimes in particularly high doses. There are miscellaneous factors contributing to the observed hemodynamic instability of ICU patients, including hypovolemic, distributive, cardiogenic or obstructive shock, MV, sedation and drugs[45,46]. It is worth mentioning that in the COVID-19 era a specific cause leading to cardiogenic shock may be related to myocardial injury caused by SARS-CoV-2. Cardiovascular involvement in COVID-19 may manifest in the form of myocarditis and pericarditis, but also as arrhythmias, acute coronary syndromes and stress-induced cardiomyopathy. When severe, all of these cardiovascular manifestations may potentially lead to acute heart failure and shock, especially in vulnerable patients with pre-existing cardiovascular disease[47-49]. Regardless of the cause, hemodynamic instability results in decreased splanchnic blood flow, leading to inadequate blood supply to the liver and subsequent liver ischemia. The resultant decreased end-organ perfusion is exaggerated in cases of hypovolemia[50].

Moreover, in the setting of passive liver congestion due to right heart failure of various etiologies, the superimposed elevated hepatic venous pressures further impair hepatic circulation by reducing the gradient between portal and hepatic venous pressures, which primarily drives the flow within the portal venous system. On top of that, the liver autoregulatory mechanisms are disrupted and become maladaptive, while the hepatic arterial buffer system fails to sustain hepatic blood flow under conditions of low mesenteric perfusion and cannot compensate for the changes in liver blood supply caused by low blood pressure, thus placing the liver at risk for further ischemic injury[51,52].

It needs to be emphasized that, although vasopressors can have beneficial hemodynamic effects in terms of restoring and maintaining hemodynamic stability and supporting vital functions, they are not void of adverse effects, which can often be detrimental. Indeed, they can cause excessive vasoconstriction and hence impair tissue perfusion through reduction of blood flow in vasoconstricted vascular beds, while at the same time they may exert deleterious effects on cardiac, metabolic, microbiome and immune function[53-55].

From a cellular standpoint, the sudden and profound reduction in systemic blood pressure, in conjunction with increased hepatic venous pressures, establish a low-flow state, which can result in the so-called hypoxic hepatocellular injury characterized by prominent centrilobular hepatocellular necrosis, since the central areas of the liver (commonly referred to as zone 3) are more susceptible to ischemic insults[51]. Mitochondrial damage and DNA fragmentation are the principal mechanisms implicated in the process of injury during hypoxic ALI, as evidenced by elevated plasma levels of glutamate dehydrogenase and cytochrome c oxidase mitochondrial DNA on the one hand and elevated

Table 1 Studies examining coronavirus disease 2019 induced acute liver injury in intensive care unit patients

Ref.	ALI definition	Number of ICU patients	ALI incidence	Major findings/outcomes
Roedl *et al*[28], 2021, Germany, Single-center, retrospective	↑ Transaminases > 20 × ULN or BIL ≥ 2 mg/dL	72	31%	ALI pts experienced higher mortality (83% for hepatocellular, 66% for mixed and 60% for cholestatic ALI), developed respiratory failure more frequently and had higher need for MV, ECMO, vasopressors and RRT
Huang *et al*[29], 2020, China, Single-center, retrospective	↑ALT > 20 × ULN	51	5.88%	ALI was not rare and was associated with high mortality due to MOF
Salik *et al*[30], 2021, Turkey, Single-center, retrospective	↑ALT/AST > 3 × ULN and/or ↑TBIL > 2 × ULN`	533	8.6%	ALI pts had higher total, 7-d and 28-d mortality, as well as higher SOFA score. ALI was more frequent in males
Arentz *et al*[31], 2020, United States, Single-center, case series	ALT or AST levels > 3 × ULN	21	14.3%	67% mortality for the overall cohort, no mortality data for ALI pts. All ALI pts (3) were on MV. High rate of cardiomyopathy in the overall cohort
Martinot *et al*[32], 2021, France, Single-center, retrospective observational cohort	AST ≥ 3 × ULN	153	9.8%	Factors associated with higher risk for ALI: Age > 75, cancer, cardiac disease, higher levels of CRP, serum Cr and Hb
Sun *et al*[33], 2020, China, Single-center, retrospective observational	↑Serum levels of liver biomarkers (*e.g.* ALT) > 2 × ULN or disproportionate ↑ ALT/AST *vs* ALP	83	18.1%	The study focused on AGI, defined as various grades of malfunction of the GI tract, and not on ALI. Pts with worse AGI grades had higher 28-d mortality, higher incidence of septic shock and worse clinical variables
Cardoso *et al*[34], 2020, Portugal, Single-center, case series	Any elevation of at least one liver test (AST, ALT, ALP, GGT, TBIL) above ULN	20	100%	Liver injury was frequent, but transient and non-severe. Late cholestasis was mainly observed
Shousha *et al*[35], 2021, Egypt, Multi-center, prospective cohort	↑Transaminases > 3 × ULN	122 ICU pts. Overall cohort consisted of 547 pts	4.91% or 3.70% (based on AST or ALT levels respectively). Data represent overall (not ICU) pts cohort	FIB-4 on admission was significantly higher in pts admitted to the ICU, those with more severe COVID-19 and non-survivors. FIB-4 score > 3.25 and ICU admission were significant predictors of mortality
Currier *et al*[36], 2021, United States, Single-center, retrospective	ALT/AST > 60, ALP > 150, or BIL > 1.5	No data available for ICU pts. Overall cohort consisted of 8028 pts	45%. Data represent overall (not ICU) pts cohort	Black COVID-19 pts with liver test abnormalities were at greater risk for ICU admission and intubation compared to other races, but non-Black pts with liver test abnormalities were at increased risk of death
Rabiee *et al*[37], 2020, United States, Multi-center, observational cohort	ALT ≥ 2 × ULN (moderate: ALT 2-5 × ULN, severe: ALT > 5 × ULN)	30 ICU pts. Overall cohort consisted of 81 hospitalized LT recipients	34.6%. Data represent overall (not ICU) pts cohort	ALI was associated with higher risk for ICU admission and higher mortality. Hispanic ethnicity, metabolic syndrome, use of vasopressors, antibiotic use and younger age were independent risk factors for ALI. ICU pts were more likely to have their immunosuppression therapy modified
Roncati *et al*[38], 2022, Italy, Case report	N/A	2	100%	Fulminant herpetic hepatitis developed as a superimposed opportunistic infection due to tocilizumab-induced immunosuppression. Both pts died due to acute liver failure
Bütikofer *et al*[39], 2021, Switzerland, Single-center, retrospective cohort	Cholestatic ALI: ALP ≥ 1.5 × ULN and GGT ≥ 3 × ULN (termed severe if additionally TBIL ≥ 2 × ULN)	34	59% (27% severe)	Pts with severe ALI had higher mortality, significantly longer ICU stay with a more complicated course and required higher levels of support
Wendel-Garcia *et al*[40], 2022, Switzerland, Single-center, prospective observational cohort (post-hoc analysis)	Acute cholestatic liver injury: ALP > 1.5 × ULN and GGT > 3 × ULN (severe if additionally BIL > 2 × ULN)	243	47%	Pts who received ketamine had an increased risk of developing cholestatic liver injury than pts who didn't. Ketamine infusion demonstrated a dose-dependency and duration-dependency association with increasing BIL and ALP levels, but it was not associated with increased hospital mortality

AGI: Acute gastrointestinal injury; ALI: Acute liver injury; ALT: Alanine transaminase; ALP: Alkaline phosphatase; AST: Aspartate transaminase; BIL: Bilirubin; COVID-19: Coronavirus disease 2019; Cr: Creatinine; CRP: C-reactive protein; ECMO: Extracorporeal membrane oxygenation; FIB-4: Fibrosis-4 index; GI: Gastrointestinal; GGT: Gamma-glutamyl transferase; Hb: Hemoglobin; ICU: Intensive care unit; LT: Liver transplant; MOF: Multiple organ failure; MV: Mechanical ventilation; Pts: Patients; RRT: Renal replacement therapy; SOFA: Sequential organ failure assessment; TBIL: Total bilirubin; ULN:

Upper limit of normal.

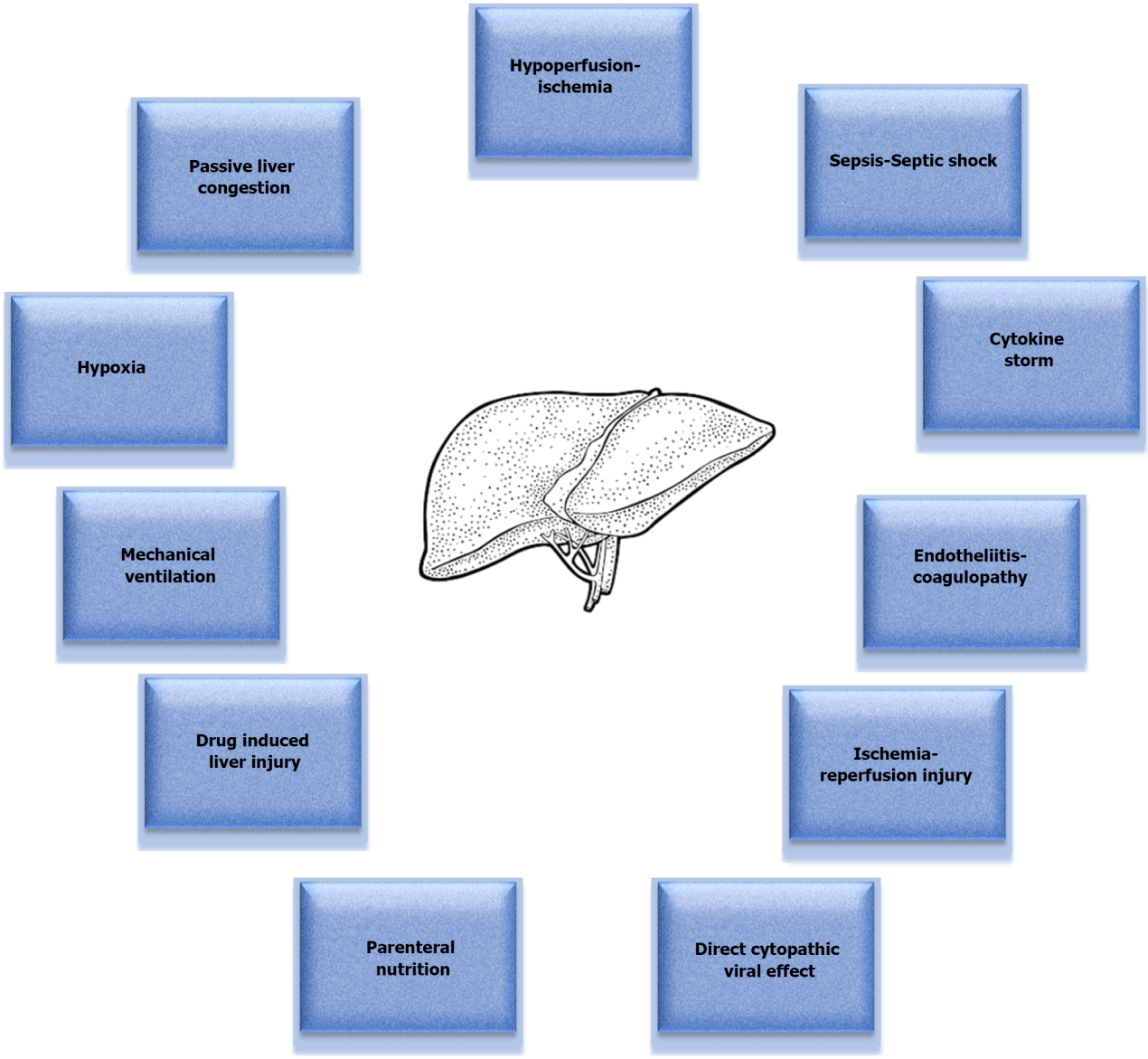

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Figure 1 Pathophysiological mechanisms implicated in the development of coronavirus disease 2019 acute liver injury in the intensive care unit setting. Figure 1 depicts the various pathophysiological mechanisms that come into play in the setting of coronavirus disease 2019 (COVID-19) infection, which eventually culminate in the development of acute liver injury (ALI) in intensive care unit (ICU) patients. Hypoperfusion and ischemia due to the frequently observed hemodynamic instability in the ICU patients play a major role in the process. Passive liver congestion as a result of congestive heart failure of various etiologies is another key determinant. The situation can be further aggravated by ischemia-reperfusion injury. Sepsis can also cause hepatic dysfunction, which is usually cholestatic in nature and reversible, but, when it progresses to septic shock, it results in multiple organ dysfunction syndrome and liver hypoperfusion, manifesting as shock liver. Besides, hypoxia due to respiratory failure and acute respiratory distress syndrome may also lead to ALI. Paradocixally, mechanical ventilation *per se* can exert deleterious effects on the liver through positive pressure ventilation, especially when accompanied by the application of high positive end-expiratory pressure and large tidal volumes. Furthermore, ALI can stem from the inflammatory cascade generated by the cytokine storm, while it can be further complicated by impaired microcirculation and thrombosis as a result of endotheliitis with concomitant coagulopathy. Drug-induced liver injury, as well as parenteral nutrition, must also not be overlooked as potential causative factors. Finally, liver injury could also be attributed to a direct cytopathic viral effect. There seems to be a complex interplay among these numerous underlying mechanisms, which may act either independently and cause COVID-19 induced ALI in the ICU setting, or more frequently synergistically and generate a relentless ALI vicious cycle.

circulating levels of nuclear DNA fragments on the other[56].

Even after restoring hemodynamic stability with the use of vasoactive drugs, it should be kept in mind that ischemia reperfusion injury may ensue, whereby reperfusion after prolonged ischemia can trigger a cascade of molecular mechanisms, paradoxically begetting further liver injury. This cascade of events involves the recruitment and activation of cellular mediators, the engagement of the complement

system, the generation of reactive oxygen species and the release of a wide variety of various cytokines, chemokines, adhesion molecules and other chemical mediators, which lead to microvascular alterations, acute inflammatory responses, derangements in microcirculation and increased hepatocellular apoptosis and necrosis[57].

Sepsis-Septic shock

In the ICU environment, sepsis represents a relentless plague with grave consequences for the critically ill patients, accounting for a significant proportion of the observed high morbidity and mortality rates. This becomes even more relevant for COVID-19 patients hospitalized in the ICU, given the fact that they require extended ICU stay with prolonged periods of invasive MV and vasopressor support, in the setting of a severely compromised immune system with a markedly dysregulated host response to infection. Sepsis may stem from multiple sources of infection and invariably results in organ dysfunction[58]. With regard to the liver, sepsis can cause hepatic dysfunction, which primarily manifests as sepsis-associated cholestatic dysfunction and to a lesser extent as hypoxic hepatitis. Sepsis, especially when caused by infections from gram-negative bacteria, increases intestinal permeability and results in endotoxin translocation from the intestinal lumen into the portal circulation. In the liver, endotoxins mount an inflammatory response through activation of Kupffer cells and macrophages, which results in secretion of proinflammatory cytokines[21,59]. This inflammatory reaction induces changes in the architecture and function of hepatocytes and cholangiocytes, leading to dysregulation of the liver metabolic signaling pathways, repression of the hepatobiliary transporter systems and decrease in canalicular contractility. All these result in bile acid retention with defective bile acid uptake, impaired bile production and secretion, inhibition of bile flow and formation of biliary sludge. The condition is further exacerbated by the impaired hepatic microcirculation due to sepsis-induced microvascular endothelial injury resulting in coagulopathy and microthrombi formation[21,60]. In the majority of critically ill patients, this type of cholestasis is reversible, but, in rare cases, destruction of the biliary epithelium due to ischemia and inflammation may cause irreversible biliary damage, progressing to secondary sclerosing cholangitis with formation of biliary casts and scarring of the bile ducts[60,61].

When sepsis culminates in septic shock, hemodynamic instability prevails, necessitating administration of vasopressors or inotropes[62]. Under these circumstances, the imminent end-organ hypoperfusion may eventually lead to shock liver in the context of multiple organ dysfunction syndrome. Indeed, the impaired hepatic perfusion leads to hypoxic hepatitis by causing direct hepatocellular injury. Despite the associated hyperdynamic state with increased cardiac output, septic shock is characterized by ongoing liver ischemia owing to impaired tissue oxygen extraction or, in other words, inability of hepatocytes to utilize oxygen. Once hepatocytes, especially those residing in the centrilobular regions of the liver, are deprived of oxygen, they start malfunctioning[21,63]. In fact, progressive tissue hypoxia promotes a catabolic state of anaerobic metabolism, suppresses mitochondrial energy production, compromises cellular membrane integrity due to loss of energy-dependent ion pumps and causes significant structural and functional abnormalities to hepatocytes[63]. The final result is direct hepatocellular damage, which is clinically described as hypoxic or ischemic liver injury and characterized by a massive, acute and transient rise in serum levels of aminotransferases[64].

Hypoxia

One of the cardinal features of COVID-19 critical illness is severe systemic hypoxia due to respiratory failure or ARDS[65]. Regardless of hemodynamic status, severe hypoxia *per se* can lead to ALI[66]. As a matter of fact, a pronounced and prolonged imbalance between oxygen supply and expenditure, secondary to profound hypoxia, can result in oxygen deprivation to the liver and thus trigger hypoxic hepatitis[64]. Hypoxia has long been implicated in the pathogenesis of liver diseases[67]. It has been shown that hypoxia induces alterations in gene expression by effectuating the activation of hypoxia-inducible factor 1α and nuclear factor kappa B, which in turn stimulate angiogenesis, chronic inflammation and epithelial-mesenchymal transition. Accordingly, the activation of these transcriptional pathways promotes liver fibrosis and increases hepatic vascular resistance, thereby diminishing liver blood flow and further aggravating liver hypoxia[68].

In most ICU cases, hypoxia, the predominant characteristic of ARDS, mandates the use of invasive MV. During ARDS management, protective ventilation strategies are employed, which incorporate the use of low tidal volumes with permissive hypercapnia[69]. However, both hypoxia and hypercapnia have been known to induce pulmonary vasoconstriction, which in turn increases pulmonary vascular resistance and right ventricular afterload[70,71]. This may cause derangements in the subtle balance between the respiratory and cardiovascular system, eventually provoking cardiocirculatory instability, which sequentially establishes an even harsher hypoxic environment[71].

In view of the fact that severely hypoxemic patients require invasive MV, often with the application of high positive end-expiratory pressure (PEEP) levels, the strain imposed on the right ventricle by MV may elicit right ventricular failure and reduce venous return and right ventricular preload, thus further affecting hemodynamic stability[72,73]. These mechanical effects are aggravated in patients with ARDS or in mechanically ventilated patients with intrinsic PEEP due to dynamic hyperinflation. As a result, the clinical course of the patients may be complicated by acute cor pulmonale. Factors associated with a

higher risk for developing acute cor pulmonale include worse oxygenation, hypercapnia, high ventilator pressures and pneumonia-related ARDS[72].

MV

Owing to the fact that respiratory failure is the most frequent indication for ICU admission, the majority of ICU patients are mechanically ventilated. Although life-saving, MV may elicit untoward effects on the function of extrapulmonary organs, including the liver, *via* multiple interactions.

It has long been recognized that positive pressure ventilation can induce a decrease in cardiac output accompanied by a reduction in hepatic arterial blood flow, while high intrathoracic pressures can jointly incur increases in hepatic venous and inferior vena caval pressures. In parallel, liver compression by the descent of the diaphragm increases hepatic venous resistance and causes a rise in intravascular portal pressure. The constellation of these MV-triggered events can cause congestive hepatomegaly and induce hepatocellular dysfunction[74].

The aforementioned effects on liver hemodynamics can be further aggravated by the application of PEEP. Indeed, it is widely known from the literature that PEEP may increase the backpressure to liver venous outflow and cause an elevation in liver venous resistance, thus decreasing total venous return and resulting in hepatic blood pooling and liver congestion[75,76]. This decrease in venous return is further amplified in hypovolemic states[71]. Besides, in a study assessing risk factors for liver injury in critically ill patients, high levels of PEEP were found to promote hepatic dysfunction[77].

Furthermore, in a study by Schricker *et al*[78], it was found that PEEP affected liver metabolism by promoting hepatic gluconeogenesis and enhancing oxidative hepatic lipid utilization for energy coverage in the liver[78]. A parallel increase in the splanchnic oxygen extraction rate was reported in response to a decline in hepatic oxygen delivery caused by a PEEP-dependent decrease in cardiac output[78]. This mechanism has been corroborated by various human studies which have shown that increasing levels of PEEP reduced splanchnic blood flow, thereby jeopardizing oxygen delivery to the abdominal viscera; yet, splanchnic oxygen consumption was usually maintained through a compensatory increase in splanchnic oxygen extraction[79].

Moreover, the application of large tidal volumes during MV markedly increases pulmonary vascular resistance. In turn, the increased pulmonary vascular resistance may compromise the systolic performance of the right ventricle, since ejection will have to take place against an increased right ventricular afterload[71]. The ensuing right ventricular dysfunction will result in high right atrial pressures, which will then be transmitted backwards to the liver, causing hepatic congestion due to a retrograde increase in the pressures of the inferior vena cava and the hepatic veins[80].

Additionally, the use of high PEEP, high inspiratory pressures and high tidal volumes during MV may alter hepatosplanchnic perfusion by precipitating varying degrees of elevation in the intra-abdominal pressure[81-83]. As a matter of fact, MV, especially with high levels of PEEP, induces changes in pressure in the intra-abdominal compartment through transmission of increased intrathoracic pressures to the abdomen and downward displacement of the diaphragm. Should a sustained rise in intra-abdominal pressure occur, this can lead to an abdominal compartment-like syndrome, thus affecting abdominal venous return and causing a decline in cardiac output as a result of a marked decrease in right ventricular preload[84]. Concurrently, the resultant intra-abdominal hypertension may compromise liver perfusion and jeopardize the physiologic hepatic function by compressing the portal vein and causing intestinal congestion[85]. At the same time, the increased abdominal pressure is transmitted to the thoracic cavity, affecting lung volumes and respiratory mechanics and generating increases in peak inspiratory, plateau and mean airway pressures of the mechanically ventilated patients[86]. Accordingly, this may negatively affect cardiac and respiratory performance, potentially leading to cardiovascular collapse, which will further exacerbate the vicious cycle of poor tissue perfusion and organ dysfunction[84]. The clinical importance of this phenomenon may be further magnified by the presence of causal factors which predispose to additional elevations of intra-abdominal pressure, such as acidosis, coagulopathy, sepsis, MV, use of PEEP or presence of auto-PEEP, pneumonia, prone positioning, aggressive fluid resuscitation or increased severity scores[87].

Finally, MV *per se* can induce an early systemic inflammatory reaction, governed by an increased expression of soluble adhesive molecules and cytokines, along with a concomitant activation of the neurohumoral axis[88].

Collectively, it becomes evident from the above that MV may exert negative effects on liver function in terms of both mechanics and hemodynamics, as well as from a neurohumoral and metabolic perspective[89].

Cytokine storm

SARS-CoV-2 activates the host immune system at different levels and in varying degrees. In critically ill COVID-19 patients, the virus triggers an uncontrolled immune response, which results in a state of generalized overt inflammation. This overwhelming inflammatory reaction is termed cytokine storm and is heralded by the excessive production of proinflammatory cytokines on the grounds of a dysregulated immune system. During this chain of events, cytokines play a pivotal role in the inflammatory cascade triggered by SARS-CoV-2. Principal cytokines involved in the process include interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α). The excessive expression of proinflammatory cytokines,

chemokines and adhesion molecules attracts neutrophils, monocytes, macrophages and platelets, which further potentiate cytokine production. The resultant overflooding of cytokines and chemokines poses a systemic stress on multiple organs, leading to tissue injury through activation of numerous signaling pathways. Ultimately, the severe systemic inflammatory response syndrome (SIRS) results in multi-organ failure. Of course, the liver is not spared throughout this process and sustains immune-mediated direct and indirect injury of varying magnitude[90-92].

It has been demonstrated that COVID-19 patients exhibit elevated levels of various inflammatory cytokines, such as IL-1β, IL-2, IL-6, IL-10, TNF-α, interferon-gamma (IFN-γ), IFN-γ-inducible protein 10, granulocyte macrophage-colony stimulating factor and monocyte chemoattractant protein-1. What is more, some of these cytokines have been shown to correlate with the severity of COVID-19 disease course. Moreover, inflammatory infiltrates, indicative of immune-mediated injury, have been found to be present in numerous tissue samples of COVID-19 patients[93]. In addition, elevated levels of IL-6 and IL-10 and low counts of CD4+ T cells have been reported to be independent risk factors for development of ALI in COVID-19 patients[94]. All the above point towards the fact that an exaggerated immune-mediated response, generating an inflammatory cascade of events known as cytokine storm, plays a key role in the development of COVID-19 induced ALI.

Endothiliitis-coagulopathy

COVID-19 is considered a thrombotic disease which affects the vessel endothelium *via* inflammation and promotes endotheliitis, coagulopathy and thrombosis[95,96]. Indeed, endotheliitis seems to be the aftermath of the complex interplay between SARS-CoV-2 and the host immune response. SARS-CoV-2 initiates an inappropriate inflammatory response that triggers the overproduction of chemical mediators and mounts cell-mediated interactions with a subsequent burst of a cytokine storm. Activated immune cells bind to the adhesion molecules expressed on the surface of endothelial cells and generate an inflammatory state. The complex and sustained activation of cellular downstream signaling pathways mediated by pro-inflammatory cytokines drives SIRS, which affects the endothelium. The integrity of the endothelial barrier is compromised, thus promoting endothelial capillary leak and interstitial oedema. Normal endothelial function is also disrupted due to the production of free radicals, the reduction of nitric oxide (NO) levels and the decrease of the endothelial NO synthase activity. Through impairment of endothelial function, NO dysregulation and increased oxidative stress lead to maldistribution of microvascular blood flow, eventually resulting in abnormal vascular tone, attenuated endothelium-dependent vasodilation, compromised tissue oxygen delivery and tissue hypoxia[97,98]. In addition, dysfunctional endothelial cells may create a procoagulant milieu, by triggering fibrin formation as well as platelet adhesion and aggregation. These derangements alter local hemorheological conditions and promote microcirculatory stasis, microthrombi formation and capillary plugging. The generalized hypercoagulable state leads to maldistribution of tissue perfusion and microcirculatory ischemia, further aggravating tissue hypoxia[97]. The reduced peripheral perfusion due to the aforementioned endothelial dysfunction affects multiple systems and may result in multi-organ failure. The liver is not left intact throughout this process and subsequently ALI emerges[98,99].

DILI

ICU patients present several unique characteristics which render them much more susceptible to the development of DILI, compared to common ward patients. First of all, ICU patients typically have multiple co-morbidities and require treatment with numerous pharmacologic agents. Oftentimes, complex drug-combination strategies are employed in an attempt to cope with infections due to multi-drug resistant microorganisms[100]. However, polypharmacy exposes them to the risk of many potential drug-drug interactions[101]. Second, their ICU stay is usually prolonged, thus mandating long-course treatment[102]. Furthermore, due to their underlying critical condition, drug pharmacokinetics are substantially modified and are therefore unpredictable, increasing DILI risk[103]. Additionally, when considering the co-existence of other causes of liver injury that are almost invariably present in ICU patients with COVID-19, it becomes evident that the risk of DILI is further accentuated.

A variety of drugs used in the ICU for the treatment of COVID-19 patients could potentially lead to liver injury. Acetaminophen is a drug widely used in the ICU with well-documented hepatotoxic properties, which may confer liver injury even at lower than maximum daily recommended doses[104]. Besides, ICU patients with COVID-19, especially those with ARDS and on invasive MV, are particularly prone to bacterial infections and other opportunistic super-infections and are therefore treated with several antibiotics and antifungals. However, many of the antibiotics prescribed in the ICU carry an inherent risk of DILI, such as penicillins, cephalosporins, fluoroquinolones, macrolides and tetracyclines [105,106]. The same holds true for antifungal agents with triazoles demonstrating the highest potential for hepatotoxicity[106,107].

Furthermore, antiviral drugs used in the treatment of COVID-19 have been implicated in the development of ALI. The effect of antiviral treatment on liver function was studied in critically ill patients with COVID-19 in a retrospective cohort study and it was concluded that the overall use of antivirals was associated with increased risk of ALI[108]. Remdesivir is the sole antiviral drug currently approved for COVID-19 treatment, although its use and benefits are still under debate[109]. Early reports from studies examining compassionate use of remdesivir indicated that hepatotoxicity was

among one of the most frequent adverse events observed during remdesivir treatment. It manifested as an increase in transaminase levels, which resulted in discontinuation of the drug in some cases[110,111]. A randomized, double-blind, placebo-controlled multicenter trial from China also reported increases in aminotransferase or bilirubin levels with remdesivir, sometimes leading to premature discontinuation of therapy[112]. However, the authors concluded that, overall, remdesivir was adequately tolerated and the serious adverse events tended to be lower in the remdesivir group than in the placebo group[112]. According to a systematic review and meta-analysis, the use of remdesivir frequently resulted in elevated transaminases with the incidence of DILI being 15.2% among COVID-19 patients receiving remdesivir[113]. Remdesivir was also found to confer an increased risk of ALI in a study analyzing data derived from VigiBase, a pharmacovigilance global database system available from the World Health Organization[114]. Indeed, the most frequently reported adverse effects related to remdesivir were increased liver enzymes, mainly transaminases and to a lesser extent bilirubin. Of note, most cases were deemed serious by virtue of requiring hospitalization or resulting in prolonged hospital stay[114]. Other studies have also shown that remdesivir can cause hepatocellular injury, but it seems that in most cases ALI is mild, asymptomatic and not clinically apparent; it is not associated with jaundice and does not progress to severe liver damage or failure, while it is characterized by low discontinuation rates and reversibility after discontinuation[115-119]. Nevertheless, remdesivir's use in the ICU is strictly limited in patients with severe COVID-19 who are not on MV[120].

Apart from antiviral drugs, immunomodulatory therapies have been put forward as therapeutic options for COVID-19. Tocilizumab, which is an anti-IL-6 receptor monoclonal antibody, is among the most utilized ones. It has been used in patients with respiratory deterioration and high oxygen requirements and has been associated with a reduction in mortality and need for intubation, when combined with corticosteroid use and administered early in the disease course[121-123]. However, its use has been associated with the development of a hepatocellular pattern of DILI, characterized by a mild to moderate elevation of transaminases in most cases[124-126]. The observed hepatotoxicity has been reported to be dose-dependent and generally transient[119]. Moreover, owing to the fact that tocilizumab induces immunosuppression, it should be used with caution, since it can increase the risk of infectious complications and may cause reactivation of hepatitis B virus (HBV) in patients with latent infections[127,128]. Thus, clinicians should always keep in mind that tocilizumab-induced ALI could also potentially arise from HBV reactivation[14].

Furthermore, low molecular weight heparins are among the most frequently used regimens in the ICU. Heparin-induced hepatotoxicity has been described in the literature, but is generally mild, transient and self-limited and does not warrant discontinuation of heparin therapy, which is an essential component of COVID-19 treatment[118,119,129-131]. Likewise, the use of systemic corticosteroids, which are routinely recommended in mechanically ventilated patients with COVID-19 and ARDS, has been rarely associated with DILI[119,120,132]. Moreover, amiodarone, which is frequently used in the ICU setting as an antiarrhythmic drug to terminate supraventricular and ventricular tachyarrhythmias, is well-known for its hepatotoxic effects and could thus contribute to the development of DILI[133].

Lastly, the use of anesthetic drugs deserves specific mention, since COVID-19 pandemic has posed unique challenges to intensivists with regard to sedative strategies. Oftentimes, achieving satisfactory and deep sedation in invasively mechanically ventilated patients has been proven problematic, since an inordinate resistance to standard sedative regimens has been typically observed. In order to overcome this impediment, ICU physicians have been compelled to resort to alternative methods of sedation[134]. As a result, ketamine has been increasingly used as a second-line anesthetic agent for long-term sedation, in combination with the standard course of sedative and analgesic treatment. However, there have been several reports associating the prolonged infusion of ketamine in high doses with cases of hepatotoxicity[40,135-138]. The resulting ALI manifests in the form of cholestatic liver injury, which may further progress to the development of secondary sclerosing cholangitis. In general, ketamine infusion requires prolonged period of administration and high total cumulative doses in order to exert its cholangiotoxic effect[40,135]. The postulated mechanism through which ketamine induces or exacerbates cholestatic liver injury includes bile stasis, which promotes the precipitation of the water-insoluble norketamine (the main active metabolite of ketamine) within the biliary tree, ultimately resulting in biliary tract dysfunction and leading to biliary strictures, biliary obstruction, cholangitis or even secondary biliary cirrhosis[40,136]. In brief, the blockade of the N-methyl-D-aspartate (NMDA) receptors in smooth muscle cells by ketamine favours bile stasis and bile duct dilation, while the ketamine-induced contraction of the sphincter of Oddi increases flow resistance within the biliary tree, thus further aggravating bile accumulation. Meanwhile, bile stasis is additionally exacerbated by gall bladder dyskinesia, which is caused by the ketamine-mediated blockade of NMDA receptors in the dorsal motor nucleus of the vagal nerve[40]. These phenomena collectively establish the ideal predisposing conditions for the precipitation of norketamine and the resultant biliary tract injury. Notably, the biliary system sustains multiple assaults, since the aforementioned sequential events act in concert with other COVID-19 related direct and indirect insults to the biliary tract, such as hypoxia, ischemia, hypoperfusion, hemodynamic instability, MV, SIRS or the virus SARS-CoV-2 itself[135,139,140].

Parenteral nutrition

Parenteral nutrition is often initiated in ICU patients in order to cover their metabolic demands in case of intolerance or contraindications to enteral feeding or whenever caloric targets are not met by enteral nutrition alone[141]. However, it has been demonstrated that ICU patients receiving total parenteral nutrition run a significantly greater risk of developing ALI than those receiving enteral nutrition[142]. Indeed, parenteral nutrition has been associated with liver injury, resulting in elevations of all liver enzymes, namely transaminases, ALP, GGT and bilirubin. Since parenteral nutrition by-passes the gut and results in decreased luminal content, it eliminates the hepatoprotective gut-derived signals, alters the enterohepatic circulation of bile acids and disrupts the normal crosstalk between the gut and the liver. Through various complex signaling pathways, parenteral nutrition causes cholestasis, steatosis, altered glucose and fat metabolism and hepatic fibrosis. Moreover, it interrupts gut mucosal integrity, causes derangements in the gut microbiota, promotes bacterial translocation and induces gut inflammation as well as increased cytokine release, all of which may contribute to further liver injury[143,144].

In addition, the lack of enteral feeding suppresses the secretion of cholecystokinin, gastrin and peptide YY. This leads to reduced intestinal motility, attenuated gallbladder contraction and decreased stimulation of bile flow, thus establishing the ideal environment for bacterial overgrowth, bile stasis, biliary sludging and subsequent bile duct obstruction. These conditions render the hepatocytes more susceptible to both direct and indirect toxic effects[144,145].

Besides, parenteral nutrition *per se* can be hepatotoxic through its components, mainly soy-derived phytosterols, manganese, aluminium and copper. Soybean oil-based lipid emulsions contain predominantly ω-6 polyunsaturated fatty acids which possess proinflammatory properties and lead to Kupffer cell activation, while phytosterols impede bile acid transport to the liver by antagonizing bile nuclear receptors. Furthermore, lipid emulsion infusion may be rarely complicated by fat overload syndrome with deleterious effects to the liver and other systemic organs[144,145].

Another potential risk of parenteral nutrition is energetic overfeeding, which increases the hazard for hepatobiliary complications[146]. Similarly, the strategy of combining parenteral with enteral nutrition, that may sometimes be employed to optimize nutritional intake in ICU patients, carries the risk of overfeeding, which predisposes to hepatic steatosis and hepatitis[141,145].

On the other side of the spectrum lies underfeeding of ICU patients, which may be related to various factors, such as feeding intolerance, hemodynamic instability, underestimation of caloric needs, frequent feeding interruptions due to diagnostic procedures or therapeutic interventions[147]. Underfeeding may contribute to a decrease in serum albumin concentration, which is a frequent underlying finding in ICU patients. Albumin levels reflect the liver's synthetic function and hypoalbuminaemia is associated with worse outcomes in critically ill patients[148].

Direct cytopathic viral effect

It has been postulated that ALI may be caused by direct viral invasion, infection and damage of hepatocytes. On this account, SARS-CoV-2 may exert direct cytopathic effects on hepatic cells, by causing lysis or promoting apoptosis and necrosis[18]. This hypothesis has been supported after identifying typical ultrastructural features and histopathological lesions of viral infection in postmortem liver biopsies of 2 cases with elevated transaminases[149]. However, the low expression level of angiotensin converting enzyme 2 (ACE2) receptors on the surface of hepatocytes, as opposed to the enriched ACE2 expression in cholangiocytes, could not support the theory of SARS-CoV-2 hepatotropism; instead, it rather implied that cholangiocytes could be targeted by SARS-CoV-2 or that alternative receptors on hepatocytes other than ACE2 could serve as the cell entry points of the virus [150]. Subsequent research revealed the presence of three SARS-CoV-2 interacting host receptors in different parts of the liver tissue, namely ACE2, transmembrane serine protease 2 and paired basic amino acid cleaving enzyme (FURIN), thus endorsing the possibility that SARS-CoV-2 may actually cause direct cytopathic injury to hepatocytes[151]. To date, the direct cytopathic viral effect has not been firmly established yet, whereas in the ICU setting this proposed mechanism does not seem to play a prominent role in the observed ALI.

It needs to be emphasized that, although there are several viruses displaying some form of hepatotropism, there may be considerable heterogeneity among them. This could be due to the fact that the immune responses mounted by the host against the virus may differ significantly depending on whether the virus is cytopathic or not, as well as on which immune evasion mechanisms are adopted by the virus, in conjunction with other factors, like impaired immunity or high viral load[92]. Furthermore, the pattern of the observed changes in transaminases during SARS-CoV-2 infection differs from the liver injury pattern of other epidemic viruses, which result in a much steeper curve of aminotransferase elevations owing to massive parenchymal necrosis[18].

Given that the mechanisms of COVID-19 induced ALI still remain largely unclear, our current understanding is limited with regard to the exact pathophysiology behind the liver injury caused by SARS-CoV-2 and how this differs or resembles the effects of other hepatotropic viruses. Even more so, data regarding the potential discrepancies in the hepatotropism of the different SARS-CoV-2 strains are lacking; hence, it would be intriguing for researchers to investigate the possible different effects that are exerted by different strains of SARS-CoV-2 on the liver and identify any associated variations in the

pathophysiology and clinical course of ALI.

LIVER HISTOPATHOLOGY IN COVID-19: IS THERE A LINK WITH PATHOPHYSIOLOGICAL MECHANISMS IN ALI?

It could be assumed that the underlying pathophysiological mechanism of ALI might be postulated based on the findings of pathological studies. However, such studies are generally inconclusive, since a firm and indisputable cause of liver injury cannot be safely deduced from histopathological reports.

As a matter of fact, histopathological findings from postmortem liver biopsy specimens have shown moderate microvesicular steatosis and mild lobular and portal activity[152]. These findings could be compatible either with direct SARS-CoV-2 infection of the liver or with DILI, but they are not conclusive in terms of supporting a definite cause of liver injury. Besides, the concomitant finding of overactivated T cells in the peripheral blood, with increased expression of proinflammatory markers and high concentration of cytotoxic granules, most likely points towards an immune-mediated injury rather than a direct cytopathic effect[152]. In another liver biopsy, the findings of mild vesicular steatosis and watery degeneration in some hepatocytes were attributed to ischemia and hypoxia, while the investigators also reported the presence of a few inflammatory cells in the hepatic sinuses, namely neutrophils, plasma cells and Kupffer cells[25].

Postmortem liver tissue biopsies from two COVID-19 cases with elevated transaminases have actually shown typical lesions of viral infection, suggestive of a direct cytopathic effect of SARS-CoV-2 on liver cells[149]. In particular, ultrastructural examination *via* transmission electron microscopy revealed that typical SARS-CoV-2 particles with corona-like spike structures were abundantly present in the cytoplasm of hepatocytes, indicating that the virus can both enter and replicate in hepatocytes. These infected hepatocytes showed cytopathic features, such as marked mitochondria swelling, endoplasmic reticulum dilation, decrease of glycogen granules and damage of the cell membrane. Histologically, plenty apoptotic hepatocytes were observed, as well as binuclear or a few multinuclear syncytial hepatocytes. Other findings included moderate microvesicular and mild macrovesicular steatosis, moderate focal lobular inflammation and mild portal inflammation with predominantly lymphocytic infiltrates[149].

Besides, in post-mortem needle core biopsies from livers, centrilobular sinusoidal dilation, mild lobular lymphocytic infiltration and patchy hepatic necrosis were mainly observed[153]. Sinusoidal dilation is a rather frequent non-specific finding observed in liver biopsies of terminally ill hospitalized patients and is often attributed to reduced venous ouflow in the hepatic veins as a result of passive liver congestion in the setting of congestive heart failure[13].

Finally, an Italian study of 48 postmortem liver biopsies reported histopathological findings suggestive of diffuse vascular alterations characterized by marked derangement of the intrahepatic vasculature and varying degrees of occlusive thrombosis[154]. Specifically, there was an increase in the number of portal vein branches, coupled with severe luminal dilation and wall fibrosis. This proliferative process was accompanied by partial or complete luminal thrombosis of portal and sinusoidal vessels, portal vein endotheliitis and portal vein wall fibrosclerosis. Steatosis was also observed in more than half of the specimens, while SARS-CoV-2 was detected in most of the samples either within blood clots or in the cytoplasm of endothelial cells[154].

PREDISPOSING FACTORS FOR ALI

COVID-19 severity has been reported to be one of the main risk factors for the development of ALI in COVID-19 patients[155]. Indeed, in a systematic review and meta-analysis it was found that increasing levels of COVID-19 severity were associated with a higher risk of developing ALI and more pronounced gastrointestinal symptoms[156]. ICU patients are more prone to developing liver impairment. This was evidenced by a meta-analysis which reported that biomarkers of liver function were significantly elevated in patients with severe and fatal forms of COVID-19 and could potentially portend a progression towards multiple organ failure, when combined with other hematologic, biochemical and immunological biomarkers[157]. Besides, higher incidence of ALI has been observed in patients with more extensive pulmonary lesions on computed tomography (CT) imaging reflected by higher CT scores, which in turn correlate with COVID-19 clinical severity[158,159].

Furthermore, pre-existing liver disease has been recognized as a predisposing factor for ALI, especially in critically ill patients with COVID-19, given the fact that patients with pre-existing liver disease, particularly those with cirrhosis, run a greater risk of hospitalization and mortality[160]. Metabolic-associated fatty liver disease has been shown to be an independent predictor for the development of mild and moderate ALI[161]. Likewise, in another patient series, metabolic-associated fatty liver disease, together with high body mass index, prevailed in the majority of patients with persistent liver injury[162]. However, it still remains controversial whether metabolic-associated fatty

liver disease is also a marker of disease severity, progression and mortality[161,162]. Shao *et al*[163] reported that the presence of fatty liver disease was associated with a higher risk of ALI in COVID-19 patients, while the presence of cirrhosis incurred an increased risk of disease progression[163]. Along the same lines, in another single-center retrospective study from Shanghai, low liver CT density, suggestive of fatty infiltration and steatosis, was shown to be a risk factor of liver injury, together with COVID-19 severity, male sex and medications, such as lopinavir/ritonavir, glucocorticoids and thymopeptides[164].

In their multicenter study involving 112 liver transplant recipients, Rabiee *et al*[37] found that ALI was associated with a higher risk for ICU admission and higher mortality in COVID-19 liver transplant recipients[37]. Interestingly, when compared to a matched control group of non-transplant patients with chronic liver disease, the incidence of ALI was higher in patients with chronic liver disease (47.5%) than in liver transplant recipients (34.6%). On multivariate analysis, younger age, metabolic syndrome, Hispanic ethnicity, administration of vasopressors and antibiotic use emerged as independent risk factors for ALI. On the other hand, non-Hispanic white liver transplant recipients had a lower risk of ALI. With regard to immunosuppressants, reducing tacrolimus or withholding mycophenolate did not increase the risk of ALI, while acute cellular rejection occurred in only one patient of the cohort. Overall, it was concluded that reduction in immunosuppression therapy was not associated with ALI or risk of mortality, thus immunosuppression can be safely modified if deemed necessary[37].

Several prediction models have been proposed in order to estimate the risk of liver injury. For instance, it has been reported that a prediction model incorporating plateletcrit, retinol-binding protein and carbon dioxide combining power could sufficiently predict the occurrence of ALI in patients with moderate COVID-19 in a timely manner[165]. Another risk scoring system was proposed by Shao *et al*[163] with the intention to predict ALI risk, which included three variables upon admission, namely ALT, CRP and lactate dehydrogenase[163]. In the same study, the authors noted that male patients were more likely to develop ALI. Interestingly, the presence of hypertension was found to convey an increased risk of ALI only for patients without any prior liver disease, but not for patients suffering from pre-existing chronic liver disease[163].

In a prospective cohort study, male sex, older age, diabetes mellitus and lymphopenia emerged as independent risk factors that could predict liver dysfunction among COVID-19 patients[166]. Other investigators concluded that male sex, along with high D-dimers and high neutrophil percentage, were the most important risk factors that could predict the development of ALI in COVID-19 patients[167]. Similarly, it was demonstrated that male sex and CPR were independently associated with liver injury [168], while another study found that male sex, high levels of serum CRP and a high neutrophil to lymphocyte ratio were potential risk factors for ALI[169]. A subsequent systematic review and meta-analysis corroborated the above findings by confirming that male sex and low lymphocyte count were associated with ALI occurrence[170].

Male sex has almost invariably been associated with the risk of liver injury and the severity of the disease[166-170]. The observed discrepancy between the genders may be related to genetic and hormonal factors, which result in lower viral load levels, milder grades of inflammation and better immune responses in women than men[171].

Finally, metabolic syndrome, which is a constellation of hypertension, hyperglycemia, dyslipidemia and obesity, has also been proposed as a predisposing factor for ALI[172]. In a recent retrospective cohort study, being overweight was a risk factor for liver injury, whereas obesity was associated with severe liver injury[173].

THERAPEUTIC STRATEGY

It should be emphasized that no specific therapy for COVID-19 induced ALI exists. Therefore, the therapeutic approach initially lies in prevention and is exclusively supportive once ALI ensues.

In the ICU setting, the main pillar of the overall ALI therapeutic strategy for critically ill COVID-19 patients consists of preventive measures. First and foremost, a detailed medical history should be obtained. This should include a comprehensive review of the medical background for concomitant liver diseases, other underlying comorbidities, use of drugs in the near past that are known to induce ALI, or exposure to alcohol, herbs and chemicals[22]. Next, high level of clinical vigilance is warranted. ICU physicians should perform meticulous clinical examination on a regular daily basis, or even at repeated intervals throughout the day if deemed necessary, in order to check for and recognize early signs and symptoms of impeding organ dysfunction. In parallel, COVID-19 patients should have regular lab tests performed to closely monitor liver enzymes and promptly identify any potential liver test abnormalities.

Among the precautionary measures to avoid ALI, prompt restoration of central hemodynamics is of paramount importance, since it will allow the hemodynamic stabilization of the patient in due time and prevent liver ischemia. In patients with hemodynamic instability, fluid resuscitation constitutes the mainstay of treatment in the attempt to restore hypovolemia. In general, a conservative over a liberal fluid strategy is advocated in critically ill COVID-19 patients with shock, provided that hypovolemia

has been addressed. Caution should be exercised so as to avoid volume overload, especially in patients with cardiac dysfunction. In order to achieve optimal loading conditions, a multimodal approach should be adopted, whereby fluid resuscitation should be guided by physical examination, follow-up of vital signs, lactate levels, dynamic parameters and point-of-care ultrasonography[120]. It has been proposed that a systematic approach incorporating focused echocardiography, lung scanning and abdominal ultrasound may facilitate the bedside evaluation of cardiac function, volume status and fluid responsiveness, while at the same time it can aid in the differential diagnosis of an undifferentiated shock, as well as in the detection of biliary or hepatic sepsis, intraabdominal fluid collection and other acute pathologies[174-176].

When hemodynamic stability is not achieved after fluid resuscitation, the administration of vasopressors is indicated. Norepinephrine should be the first-line vasopressor, while vasopressin is recommended as a second-line agent if hemodynamic status does not improve. In case of persistent hypoperfusion despite adequate fluid loading and use of vasopressors, initiation of intravenous inotropes, preferably dobutamine, is advised, particularly if there is evidence of cardiac dysfunction. Excessively high doses of vasopressors and inotropes should be avoided. Titration of vasoactive agents should generally target a mean arterial pressure of 60-65 mmHg. The ultimate goal is to achieve and maintain adequate end-organ perfusion by restoring arterial pressure and optimizing cardiac output [120].

Furthermore, increased awareness is warranted for the early identification and management of sepsis. Immediate control of the source of sepsis is crucial, along with the optimization of the patient's hemodynamic profile. Prompt and appropriate antibiotic therapy is the cornerstone in the management of patients with sepsis. In case of suspected infection, ICU physicians should start early antibiotic treatment, initially with an empiric broad spectrum antibiotic and subsequently modify antimicrobial therapy according to culture results and susceptibilities, by de-escalating or changing to a narrow spectrum antimicrobial which targets the specific pathogen[177]. The appropriate antibiotic regimen should be selected with caution, as several common antibiotic agents may carry a high risk for ALI or other adverse events. Moreover, the clinicians should be aware of drug-drug interactions and be alert for potential hepatotoxicity reactions[178]. In any case, judicious antibiotic stewardship is highly recommended.

Besides, the importance of using pharmacologic thromboprophylaxis in critically ill patients cannot be overemphasized. As a matter of fact, thromboprophylaxis is strongly recommended in the recently published surviving sepsis campaign guidelines on the management of adults with COVID-19 in the ICU[120].

Hypoxia should be consistently addressed and corrected by administering the minimum amount of supplemental oxygen that will achieve a target of peripheral oxygen saturation (SpO_2) between 92% and 96%. Regarding patients who are on invasive MV, specific attention ought to be paid to the implementation of lung-protective ventilation strategies. Low tidal volumes (4-8 mL/Kg of predicted body weight) should be applied, coupled with a target of plateau pressure < 30 cm H_2O and a driving pressure < 14 cm H_2O[120]. It should be emphasized that the volume- and pressure- limited ventilation strategy may lead to hypercapnia. Despite the fact that the strategy of permissive hypercapnia is frequently adopted in the ICU, clinicians should bear in mind that hypercapnia, combined with persistent hypoxia which is frequently observed in ARDS patients with COVID-19, may cause profound pulmonary arterial vasoconstriction, increase right ventricular afterload and jeopardize hemodynamic status with grave consequences for the liver[69,177]. It should also be noted that oftentimes ventilatory management needs to be individualized, given that different patient profiles exist with diverse lung mechanics and discrepant responses to a certain ventilatory strategy[179]. In these cases, the ventilatory approach will need to diverge from the conventional form of ventilation in ARDS. Along these lines, although a higher over a lower PEEP strategy is generally recommended in ARDS, clinicians should avoid using very high PEEP, since this could compromise right ventricular function and adversely affect liver function. Thus, close monitoring is advised so that impending cardiopulmonary deterioration is promptly recognized[179]. Furthermore, appropriate ventilator settings should be meticulously adjusted in order to minimize or prevent auto-PEEP, which can further aggravate right ventricular performance[72]. Prone positioning for 12-16 h *per* day is also recommended as a measure to improve hypoxia[120]. In cases of severe hypoxia with persistent patient-ventilator dyssynchrony or persistently elevated plateau pressures, neuromuscular blocking agents should be employed either as intermittent boluses or continuous infusions, as needed[120]. Finally, after other options have failed to enhance oxygenation, it is reasonable to apply rescue strategies. Alveolar recruitment maneuvers could be used with caution and under close monitoring for barotrauma and cardiovascular collapse, while a trial of an inhaled pulmonary vasodilator might also be attempted[120]. In cases of refractory ARDS with or without hemodynamic shock, veno-venous ECMO might be considered as a last resort to improve oxygenation and restore hemodynamic stability[120,179]. It is also considered prudent to monitor intra-abdominal pressure for potential persistent MV-related increases and address accordingly[86].

Besides, specific attention should be paid to sedation practices used in the ICU setting. If possible, administration of multiple sedative agents should be avoided in order to decrease potential risk of side-effects and drug-drug interactions. Moreover, sedatives should be titrated to the lowest effective doses and should not be administered for prolonged duration[134]. Daily sedation intervals should be applied

when appropriate[180]. Regarding ketamine, a strategy of withholding use of this agent is generally advised due to its reported adverse effects. If used, ketamine should be administered at the lowest possible doses and for brief time periods[135,136].

Nutritional status should be regularly assessed and early enteral nutrition should be initiated[181]. Parenteral nutrition should be refrained and used only in cases where enteral nutrition is not possible or contraindicated[181,182]. Caution should be exercised in order to avoid under- or over-feeding. Any nutrient deficiencies should be replaced[181]. Glycemic control should be optimized. Provided that it does not cause hypoglycemic episodes, early and intensive intravenous insulin therapy is encouraged in the ICU setting, since it reduces the risk of cholestatic liver dysfunction and the formation of biliary sludge[183,184].

In the event of ALI occurrence, every effort should be focused on restraining its progression and thus minimizing any ALI-associated consequences. Once ALI arises, it is important to seek and recognize the underlying etiology and address the responsible precipitating factor in a timely fashion. Currently, there are no specific therapies that mitigate or reverse COVID-19 induced ALI. Nevertheless, prompt diagnosis of ALI and initiation of supportive treatment is crucial. In cases of mild or moderate ALI, general supportive measures with close monitoring of liver chemistries should suffice and, in most cases, ALI will eventually subside[185].

Infrequently, cases of severe ALI may occur. In such a scenario, supportive treatment should aim at preserving liver perfusion and microcirculation, by optimizing circulatory and respiratory conditions, relieving liver congestion, restoring electrolyte and acid-base balances and eliminating any underlying precipitating factor. If DILI is suspected, the administration of the causative pharmacologic agent should be discontinued[178]. Time of withdrawal of the culprit hepatotoxic factor is of major importance; hence, high level of clinical awareness is advised along with vigilant monitoring of liver enzymes[185]. With regard to acetaminophen toxicity, therapy with N-acetylcysteine should be promptly instituted by administering either an intravenous or an oral acetylcysteine regimen[186].

During the COVID-19 pandemic, the notion of continuous renal replacement therapy as a blood purification method to combat cytokine storm has been revived. In COVID-19 clinical practice, continuous renal replacement therapy can be used for septic patients with volume overload, patients with severe metabolic acidosis and those with electrolyte disturbances or progressive azotemia. Apart from its well-established indications, it has been proposed that continuous renal replacement therapy might have a potential role in critical COVID-19 cases with an excessive cytokine storm by removing cytokines and other inflammatory mediators[187,188]. Therapeutic plasma exchange has also been put forward as a promising adjunctive rescue therapy in the battle of COVID-19 critical illness, which could mitigate cytokine storm effects and thus reverse end-organ failure[189,190]. Finally, ECMO has been used as a salvage therapy for both respiratory and circulatory support[191].

Figure 2 depicts the steps of the therapeutic approach that should be consistently followed and addressed by ICU physicians both for the prevention and treatment of COVID-19 induced ALI.

CONCLUSION

Despite the conflicting estimates of its prevalence, ALI represents a common and often under-recognized complication of COVID-19 in ICU patients that deserves more clinical attention, considering the fact that it is intertwined with significant clinical ramifications and poor patient outcomes. COVID-19 induced ALI may present with varying degrees of clinical severity. Increased clinical vigilance is therefore advised, since it can easily be overlooked. In the ICU setting, several pathophysiological mechanisms may be implicated in the development of COVID-19 induced ALI. These may act either independently or more frequently synergistically. In the latter case, the effect exerted on the liver is cumulative, thus increasing the severity of ALI. Therefore, it is of utmost importance for ICU clinicians to ensure that they consistently comply with certain predefined bundles of preventive and therapeutic measures in order to alleviate the burden of COVID-19 associated ALI in ICU patients. Ongoing research on the particular scientific field will further elucidate the pathophysiology behind ALI and address unresolved issues, in the hope of mitigating the tremendous health consequences imposed by COVID-19 on ICU patients.

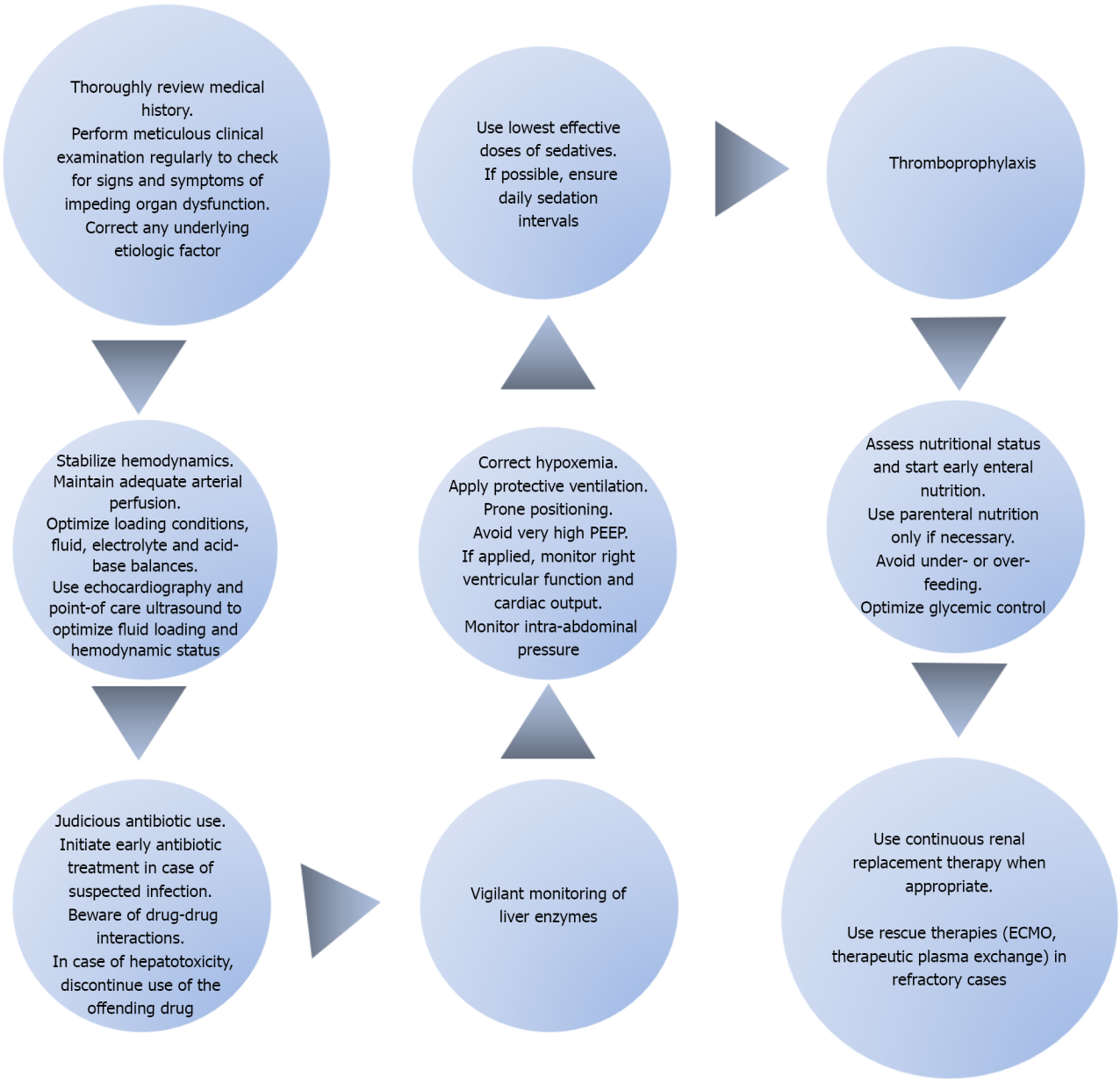

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Figure 2 Steps of the intensive care unit therapeutic approach in patients with coronavirus disease 2019 induced acute liver injury. The intensive care unit (ICU) therapeutic approach for coronavirus disease 2019 patients with acute liver injury (ALI) lies in preventive measures and is exclusively supportive once ALI ensues. After obtaining a detailed medical history, a thorough physical examination should be performed on a regular basis. Upon recognition of any signs of impeding liver dysfunction, the possible etiologic factor should be systematically sought and appropriately addressed. Hemodynamic stabilization of the patient is of paramount importance. The goal is to maintain adequate arterial perfusion in order to prevent liver ischemia. The use of echocardiography and point-of-care ultrasonography will help determine the hemodynamic status and optimize loading conditions. It is important to constantly maintain fluid, electrolyte and acid-base balances. Furthermore, early identification and management of sepsis cannot be overemphasized. Prompt initiation of appropriate antibiotic therapy is the mainstay of sepsis management. Antibiotics should be used judiciously with an increased level of awareness for possible drug-drug interactions. In case of hepatotoxicity, the administration of the responsible pharmacologic agent should be discontinued. Close monitoring of liver enzymes is advised at all times. Furthermore, hypoxia should be consistently addressed and corrected. In mechanically ventilated patients, a lung-protective ventilation strategy should be implemented with the use of low tidal volumes coupled with a target of plateau pressure < 30 cm H_2O. The application of very high positive end-expiratory pressure should be avoided because it could compromise right ventricular performance and precipitate passive liver congestion. Moreover, prone positioning is recommended in order to improve hypoxia. Intra-abdominal pressure should also be monitored so as to prevent any potential increases related to mechanical ventilation which could jeopardize liver function. Additionally, optimal sedation strategies should be applied with the lowest effective doses and daily sedation intervals. Thromboprophylaxis is strongly recommended as a preventive measure in all ICU patients. Besides that, nutritional status should be regularly assessed and efforts should focus on initiating early enteral feeding while avoiding parenteral nutrition. Under- or over- feeding should be avoided and glycemic control should be optimized. Finally, renal replacement therapy can be used whenever indicated. In refractory cases, rescue therapies may be employed with the use of extracorporeal membrane oxygenation and therapeutic plasma exchange in an attempt to provide circulatory and respiratory support and reverse end-organ failure. PEEP: Positive end-expiratory pressure; ECMO: Extracorporeal membrane oxygenation.

FOOTNOTES

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REVIEW

Alterations of the gut microbiota in coronavirus disease 2019 and its therapeutic potential

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Abstract

The coronavirus disease 2019 (COVID-19) pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) poses a serious threat to global health. SARS-CoV-2 infects host cells primarily by binding to angiotensin-converting enzyme 2, which is coexpressed in alveolar type 2 cells and gut epithelial cells. It is known that COVID-19 often presents with gastrointestinal symptoms and gut dysbiosis, mainly characterized by an increase in opportunistic pathogens and a decrease in beneficial commensal bacteria. In recent years, multiple studies have comprehensively explored gut microbiota alterations in COVID-19 and highlighted the clinical correlation between dysbiosis and COVID-19. SARS-CoV-2 causes gastrointestinal infections and dysbiosis mainly through fecal-oral transmission and the circulatory and immune pathways. Studies have shown that the gut microbiota and its metabolites can regulate the immune response and modulate antiviral effects. In addition, the gut microbiota is closely related to gastrointestinal symptoms, such as diarrhea, a common gastrointestinal symptom among COVID-19. Therefore, the contribution of the gut microbiota in COVID-19 should not be overlooked. Strategies targeting the gut microbiota *via* probiotics, prebiotics and fecal microbiota transplantation should be considered to treat this patient population in the future. However, the specific alterations and mechanisms as well as the contributions of gut microbiota in COVID-19 should be urgently further explored.

Key Words: COVID-19; SARS-CoV-2; Angiotensin-converting enzyme 2; Gut microbiota; Dysbiosis; Lung

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Core Tip: Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has become a global health threat. SARS-CoV-2 infects host cells through binding to angiotensin-converting enzyme 2. COVID-19 patients exhibit gut dysbiosis. Here, the gut microbiota alterations in COVID-19 are summarized. The pathways and possible mechanisms of dysbiosis caused by SARS-CoV-2, as well as the impact of the gut microbiota and its metabolites on the inflammatory response and antiviral effects during the course of the disease are also described. Therefore, targeting the gut microbiota should be considered a promising strategy for COVID-19 prevention, treatment, and prognostic assessment.

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INTRODUCTION

Coronavirus disease 2019 (COVID-19) is a new acute infectious disease that is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which belongs to the *Coronaviridae* family[1]. SARS-CoV-2 infects host cells through the binding of the S protein to angiotensin-converting enzyme 2 (ACE2) and interacts with transmembrane serine protease 2 (TMPRSS2), which cleaves the viral S-protein, allowing efficient viral fusion[2,3]. Interestingly, ACE2 is coexpressed in alveolar type 2 cells and intestinal and colonic epithelial cells, especially in small intestinal enterocytes[4,5]. Correspondingly, in addition to infecting the respiratory system and causing respiratory symptoms (*e.g.*, fever, dry cough, dyspnea, myalgia, headache, *etc.*), SARS-CoV-2 also infects the gastrointestinal tract, where it replicates abundantly[6]. Overwhelming evidence substantiates the detection of viral RNA in fecal samples or rectal swabs from COVID-19 patients[6-8]. It is estimated that up to 48.1% of COVID-19 patients had fecal samples positive for viral RNA[7], even when the virus was not detected in respiratory and/or sputum samples[9]. Moreover, researchers have found that the gut microbiota composition of COVID-19 patients exhibits significant alterations (dysbiosis), mainly characterized by an increase in the abundance of opportunistic pathogens and a decrease in the abundance of beneficial commensal bacteria[10]. Gut dysbiosis is closely associated with gastrointestinal symptoms and disease severity[11,12]. Therefore, the crosstalk between the gut microbiota and COVID-19 is gaining attention.

The gut microbiota has become a hot research topic in recent years. The resident microbial composition of the human gut mainly includes bacteria, archaea, viruses and fungi[13]. The human gut microbiota consists of more than 10^{14} bacteria and comprises approximately 500 to 1000 species. Gut bacteria in healthy individuals are mainly *Actinobacteria*, *Firmicutes*, *Proteobacteria*, and *Bacteroidetes*[13]. The complex gut microbiota communities have important genomic and enzymatic properties and perform a critical role in the immune system, which protects against pathogens and helps maintain gut microbiota homeostasis. Gut microbiota homeostasis is essential for maintaining human health. Conversely, dysbiosis can lead to metabolic disturbance, immune dysfunction and systemic inflammation and has been linked to various diseases[14].

Therefore, this review mainly summarizes the gut microbiota alterations and the possible mechanisms of dysbiosis in COVID-19. Furthermore, we highlight the theoretical basis that the gut microbiota can be considered a promising therapeutic target in COVID-19, potentially interfering with immune and inflammatory responses and antiviral effects. Finally, we also reviewed multiple interventions targeting the gut microbiota, such as prebiotics, probiotics and fecal microbiota transplantation (FMT), which could optimize COVID-19 treatment.

GUT DYSBIOSIS EXISTS IN COVID-19 AND IS ASSOCIATED WITH DISEASE SEVERITY

Gut dysbiosis in COVID-19 patients has received widespread attention in recent years (Table 1)[10,12,15-22]. The gut microbiota is significantly altered in COVID-19 patients receiving and not receiving medication compared to that in non-COVID-19 individuals[12]. Gut dysbiosis persists throughout the course of the disease and even after viral clearance[10,12]. Yeoh *et al*[12] suggested that members of the *Bacteroidetes* phylum and *Actinobacteria* were predominant in COVID-19 and non-COVID-19 individuals, respectively. In an American cohort study, *Peptoniphilus*, *Corynebacterium*, and *Campylobacter* were identified as the most enriched genera in COVID-19 patients[23]. Immunomodulatory gut bacteria, such as *Faecalibacterium prausnitzii* (*F. prausnitzii*), *Bifidobacterium adolescentis* and *Bifidobacterium longum*, were depleted in COVID-19, and their depletion was correlated with an elevation in the levels of inflammatory cytokines and markers (CXCL10, IL-10, TNF-α, CCL2, and CRP)[12]. However, Tao *et al*[16]

Table 1 Alterations of the gut microbiota composition in coronavirus disease 2019 patients

Study	Method	Increased abundance of gut microbiota	Decreased abundance of gut microbiota	Main conclusion
Gu *et al* [15], 2020	16S rrna	*Streptococcus*, *Rothia*, *Veillonella*, *Actinomyces*, *Erysipelatoclostridium*	*Ruminococcaceae family*, *Lachnospiraceae family*, *Agathobacter*, *Fusicatenibacter*, *Roseburia*	Gut microbiota has potential value as a diagnostic biomarker and therapeutic target for COVID-19
Zuo *et al* [10], 2020	Shotgun	*Clostridium hathewayi*, *Actinomyces viscosus*, *Bacteroides nordii*	*Eubacterium*, *Faecalibacterium prausnitzii*, *Roseburia*, *Lachnospiraceae*	Fecal microbiota alterations are associated with fecal virus levels and COVID-19 severity; symbionts were depletion and opportunistic pathogens were enrichment in COVID-19 patients; gut dysbiosis persists in COVID-19 patients after virus clearance
Tao *et al* [16], 2020	16S rrna	*Streptococcus*, *Clostridium*, *Lactobacillus*, *Bifidobacterium*	*Bacteroidetes*, *Roseburia*, *Faecalibacterium*, *Coprococcus*, *Parabacteroides*	IL-18 level was higher in the fecal samples from COVID-19 patients; dysbiosis may contribute to SARS-CoV-2-induced production of inflammatory cytokines and cytokine storm in the gut
Tang *et al*[17], 2020	Q-PCR	*Enterococcus*, *Enterobacteriaceae*	*Faecalibacterium prausnitzii*, *Clostridium butyricum*, *Clostridium leptum*, *Eubacterium rectale*	Specific gut microbiota can be considered diagnostic biomarkers for COVID-19; the Ec/E ratio can be used to predict death in critically ill patients
Zuo *et al* [18], 2020	Shotgun	*Candida albicans*, *Candida auris*, *Aspergillus flavus*	-	The guts of COVID-19 patients are accompanied by massive fungal blooms
Yeoh *et al*[12], 2021	Shotgun	*Actinobacteria*, *Ruminococcus gnavus*, *Ruminococcus torques*, *Bacteroides dorei*	*Bifidobacterium adolescentis*, *Faecalibacterium prausnitzii*, *Eubacterium rectale*	Immunomodulatory gut bacteria were depleted in COVID-19 patients; gut dysbiosis persists in COVID-19 patients after virus clearance; gut microbiota composition was associated with disease severity
Wu *et al* [19], 2021	16S rrna	*Streptococcus*, *Weissella*, *Enterococcus*, *Rothia*, *Lactobacillus*, *Actinomyces*, *Granulicatella*	*Blautia*, *Coprococcus*, *Collinsella*	Personalized microbiome affects disease outcomes in COVID-19 patients; targeting the gut microbiota has potential to prevent and treat COVID-19
Zuo *et al* [20], 2021	Shotgun	*Collinsella aerofaciens*, *Collinsella tanakaei*, *Streptococcus infantis*, *Morganella morganii*	*Parabacteroides merdae*, *Bacteroides stercoris*, *Alistipes onderdonkii*, *Lachnospiraceae bacterium 1_1_57FAA*	Elimination of gut SARS-CoV-2 activity and modulation of gut microbiome composition should be considered new treatments for COVID-19; the 3′ end of SARS-CoV-2 genome was highly covered than the 5′ end
Lv *et al* [21], 2021	ITS sequencing	-	Ascomycota (*Aspergillaceae*, *Candida parapsilosis*, *Talaromyces wortmannii*); Basidiomycota (*Malassezia yamatoensis*, *Rhodotorula mucilaginosa*, *Moesziomyces aphidis*, *Trechispora sp. and Wallemia sebi*)	Total gut fungal burden was significantly elevated in patients infected with SARS- CoV-2; altered gut fungi and microbiota are closely related to patient clinical characteristics
Suskun *et al*[22], 2022	16S rrna	*Bifidobacterium adolescentis*, *Dorea formicigenerasus*, *Eubacterium dolichum*, *Eggerthella lenta*	*Faecalibacterium prausnitzii*	First evaluate the microbiota composition in multisystem inflammatory syndrome in children cases

COVID-19: Coronavirus disease 2019; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; IL: Interleukin.

found that COVID-19 patients had higher abundances of *Clostridium*, *Veillonella*, *Streptococcus*, *Fusobacterium*, *Lactobacillus*, *Escherichia* and *Bifidobacterium* and lower abundances of *Bacteroidetes*, *Sutterella*, *Faecalibacterium*, *Coprococcus* and *Parabacteroides* than controls. The increased richness of *Streptococcus* exacerbates the risk of opportunistic pathogenic infections[24].

Moreover, it is widely thought that gut dysbiosis is closely associated with COVID-19 severity. The basal gut microbiota of healthy individuals and its alterations during SARS-CoV-2 infection influence host susceptibility to SARS-CoV-2 and disease recovery. The abundances of *Coprobacillus*, *Clostridium ramosum*, and *Clostridium hathewayi* were significantly and positively associated with COVID-19 severity, while the abundances of *Bifidobacterium bifiduz*, *Alistipes onderdonkii* and *F. prausnitzii* were negatively associated[10,12]. However, even different members of the same phylum, such as Firmicutes, have opposing correlations in influencing disease severity and regulating ACE2 expression[10]. ACE2 presents a critical role in gut microbial ecology, gut inflammation and innate immunity[25]. Moreover, the abundances of other bacteria, including *Bacteroides dorei* (*B. dorei*), *Bacteroides thetaiotaomicron* (*B. thetaiotaomicron*), *Bacteroides ovatus* (*B. ovatus*), *Ruminococcus*, *Clostridium citroniae*, *Bifidobacterium*, and *Haemophilus parainfluenzae*, were negatively associated with viral load in fecal samples from SARS-CoV-2-infected patients[10]. Interestingly, *B. dorei*, *B. thetaiotaomicron*, and *B. ovatus* can downregulate ACE2 expression in the murine gut[10,26], suggesting that Bacteroides species may play a protective role against SARS-CoV-2 infection by interfering with ACE2 production. The abundances of *Erysipelotrichaceae bacterium*, *Prevotella copri*, and *Eubacterium dolichum* have been reported to be positively correlated with fecal viral load[10,19]. Furthermore, *Prevotella*, *Enterococcus*, *Enterobacteriaceae*, and

Campylobacter can contribute to higher infectivity and worse prognosis in COVID-19[27]. For instance, *Prevotella* is associated with enhanced T helper 17 (Th17)-mediated mucosal inflammation, stimulating the production of cytokines and subsequently promoting neutrophil recruitment and inflammation[28]. Although it has been established that an altered gut microbiota composition is prevalent in COVID-19, conflicting results have been reported due to the heterogeneity of the gut microbiota itself, the sample size, and so on. Geographic and demographic differences also appear to affect the conclusions and dysbiosis recovery after SARS-CoV-2 infection[10,29]. Therefore, targeting specific gut microbiota alterations represents a potential strategy to alleviate disease severity in COVID-19.

POTENTIAL PATHWAYS AND MECHANISMS OF SARS-COV-2-INDUCED GUT INFECTION AND DYSBIOSIS

Generally, SARS-CoV-2 is principally transmitted *via* respiratory droplets and close contact, subsequently inducing diverse symptoms[30]; however, this paradigm does not explain how SARS-CoV-2 causes gut infection and dysbiosis. An *in vitro* study reported that the virus could be transmitted by the fecal-oral route through contaminated water, food, *etc*[30,31]. Since then, a new SARS-CoV-2 transmission route has been revealed (Figure 1). Notably, many viral activities are greatly diminished or even lost after passing through the gastrointestinal tract since gastric and intestinal fluids (low pH, rich in bile and digestive enzymes) can destroy the viral lipid envelope, inhibiting infectivity. For example, SARS-CoV has long been thought to be inactivated under acidic conditions (pH < 3). However, SARS-CoV-2 seems to overcome this obstacle since virus have been detected in the feces of infected individuals[30,31], and viruses isolated from feces can survive for an additional 1-2 d[32]. Therefore, SARS-CoV-2 may remain infectious in feces, particularly when patients have diarrhea[1]. Furthermore, virus-containing sputum swallowed by COVID-19 patients may be another pathway of gut infection because viscous sputum can protect the virions, preserving virus infectivity[33]. However, in the absence of evidence of fecal viral titers and viral viability in sewage and contaminated food, the capability of SARS-CoV-2 to be transmitted by the fecal-oral route requires further confirmation.

Moreover, circulatory and immune pathways are reportedly critical for SARS-CoV-2 to cause gut infection and dysbiosis (Figure 1). Studies conducted on SARS showed that coronaviruses damage lung tissue and then migrate to the systemic circulation, where they migrate to gut cells through the circulatory and lymphatic systems[34]. The virus can be found in the blood samples and gut of COVID-19 patients; in addition to those in epithelial cells, viral components are mainly present in intestinal lymphocytes and macrophages[31,35]. Therefore, it is speculated that SARS-CoV-2 may be transported from the lungs to other tissues, including the gastrointestinal tract, through transport *via* immune cells, similar to influenza virus[35,36]. Subsequently, SARS-CoV-2 invades gut epithelial cells by binding to AEC2[37], causing the release of cytokines and chemokines and triggering a gut inflammatory response characterized by neutrophil, macrophage, and T-cell infiltration, which further promotes dysbiosis[38-40]. In addition, the amino acid transport function of ACE2 is related to the microecology in the gut. B^0AT1, a molecular ACE2 chaperone, mediates the absorption of neutral amino acids in the intestinal epithelium[41]. Studies have confirmed that SARS-CoV-2-induced downregulation of B^0AT1 on gut epithelial cells contributes to gut barrier disruption and dysbiosis, promoting pathogen invasion and COVID-19 exacerbation[42-44]. de Oliveira *et al*[45] hypothesized that the internalization of SARS-CoV-2 causes ACE2 downregulation, leading to mechanistic target of rapamycin (mTOR) inhibition and intestinal autophagy activation. Interestingly, autophagy can regulate the gut microbiota, and increased autophagy has been associated with diarrhea[45,46]. There is currently no evidence, however, that the ACE2/mTOR/autophagy pathway is involved in the pathogenesis of COVID-19. Paradoxically, *Coprobacillus*, which is mostly correlated with the COVID-19 severity, has been shown to increase colonic expression of ACE2[26,47]. Therefore, the net expression of ACE2 in the gut remains largely unclear, warranting further investigation.

Misuse and overuse of antibiotics, common in initially treating COVID-19 patients[48], significantly impact the gut microbiota (Figure 1). Yeoh *et al*[12] found that COVID-19 patients treated with and without antibiotics had different gut microbiota composition. In addition, antibiotics attenuated the antiviral activity of commensal-enhanced type I interferons (IFN-I)[49]. Therefore, antibiotics are unlikely to improve patient outcomes without comorbid bacterial infections but instead exacerbate and prolong gut dysbiosis in this patient population.

IMPACT OF THE GUT MICROBIOTA AND ITS METABOLITES ON THE INFLAMMATORY RESPONSE AND ANTIVIRAL ACTIVITIES

Immune and inflammatory responses are important pathophysiological mechanisms in the pathogenesis of COVID-19. SARS-CoV-2 invades cells through ACE2 and TMPRSS2, where it replicates rapidly, producing and releasing large numbers of viruses and inducing excessive inflammatory

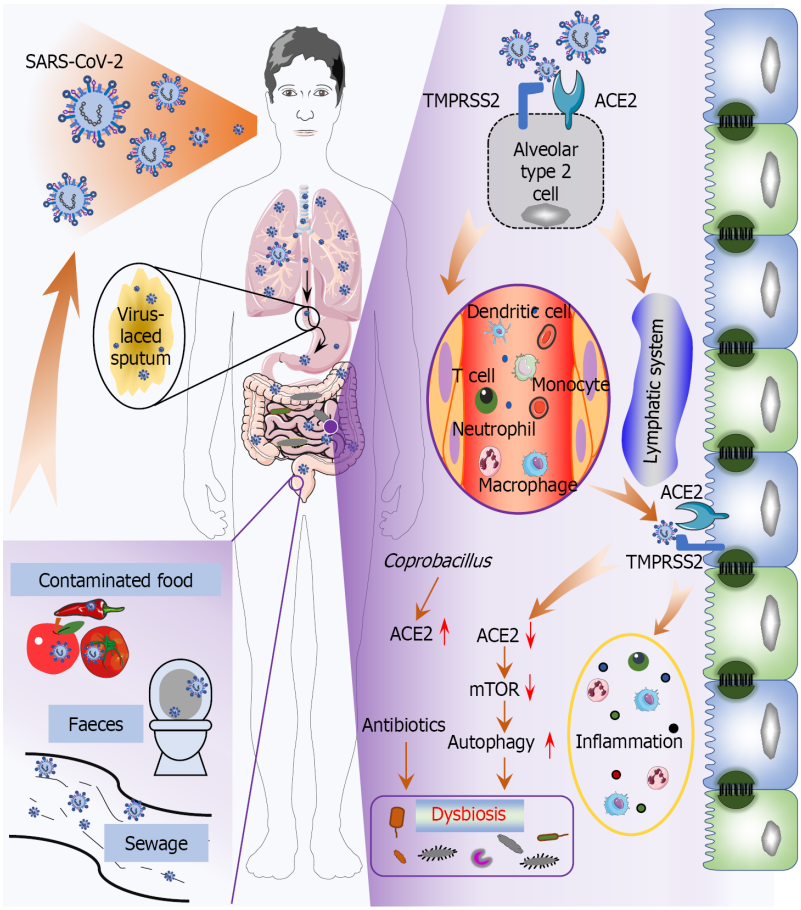

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Figure 1 Potential pathways and mechanisms of gut infection and dysbiosis induced by severe acute respiratory syndrome coronavirus 2. Fecal-oral transmission of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in contaminated water and food, or by swallowing virus-laced sputum, results in gut infection and dysbiosis. SARS-CoV-2 infects alveolar type 2 cells and damages lung tissue before invading the gut through circulating immune cells and the lymphatic system and infiltrating gut epithelial cells *via* angiotensin-converting enzyme 2 (ACE2) and transmembrane serine protease 2, subsequently triggering gut inflammation and further promoting dysbiosis. Internalization of SARS-CoV-2 Leads to downregulation of ACE2, resulting in inhibition of mechanistic target of rapamycin and subsequent activation of gut autophagy, which modulates the gut microbiome. However, gut microbiota top associations with disease severity in coronavirus disease 2019 patients, such as *Coprobacillus*, have been shown to upregulate ACE2 expression in the gut. The improper use of antibiotics can also lead to dysbiosis. TMPRSS2: Transmembrane serine protease 2; ACE2: Angiotensin-converting enzyme 2; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; mTOR: Mechanistic target of rapamycin.

cytokine release "cytokine storm", such as IL-6, IL-1β, TNF-α, and IFN[41,50] (Figure 2). Excessive production of proinflammatory cytokines is pathologically associated with acute respiratory distress syndrome, extensive tissue damage, and even death. Viral replication after SARS-CoV-2 invades cells induces immune cells to recognize and bind viral pathogen-associated molecular patterns through pattern recognition receptors (PRRs), followed by the activation of the NF-κB, IRF3 and JAK/STAT signaling pathways to induce the expression of proinflammatory factors, IFNs and IFN-stimulated genes (ISGs)[41,51,52]. In addition, killing or damage of cells by SARS-CoV-2 results in the release of danger-associated molecular patterns, activating RIG-I-like receptors and NOD-like receptors and subsequently facilitating proinflammatory factor expression[41,51]. Dysbiosis after SARS-CoV-2 infection further damages the gut barrier and promotes the production of inflammatory factors such as CXCL10, IL-2, IL-4, IL-6, IL-10, IL-18, and TNF-α[16,53,54]. For example, *B. dorei* and *Akkermansia muciniphila* were positively associated with IL-1β, IL-6 and CXCL8[12]. Subsequently, opportunistic pathogens and inflammatory factors infiltrate the circulation and cause systemic inflammation and infection[55]. Therefore, SARS-CoV-2 infection promotes gut inflammation to aggravate dysbiosis, which in turn exacerbates inflammation and disease progression, forming a vicious cycle.

The gut microbiota and its metabolites influence the host immune response, inflammation and the development and regression of pulmonary infectious diseases, such as influenza A virus and *Streptococcus* pneumonia[56]. A healthy microbiome protects against respiratory viral infections[57,58]. During respiratory viral infection, the gut microbiota regulates the host immune response *via* the gut-lung axis, which refers to the crosstalk between the gut microbiota and lung. Therefore, endotoxins and microbial metabolites can affect the lung through the blood circulation, and conversely, lung inflammation will affect the gut microbiota. It has been reported that gut dysbiosis significantly increases mortality from

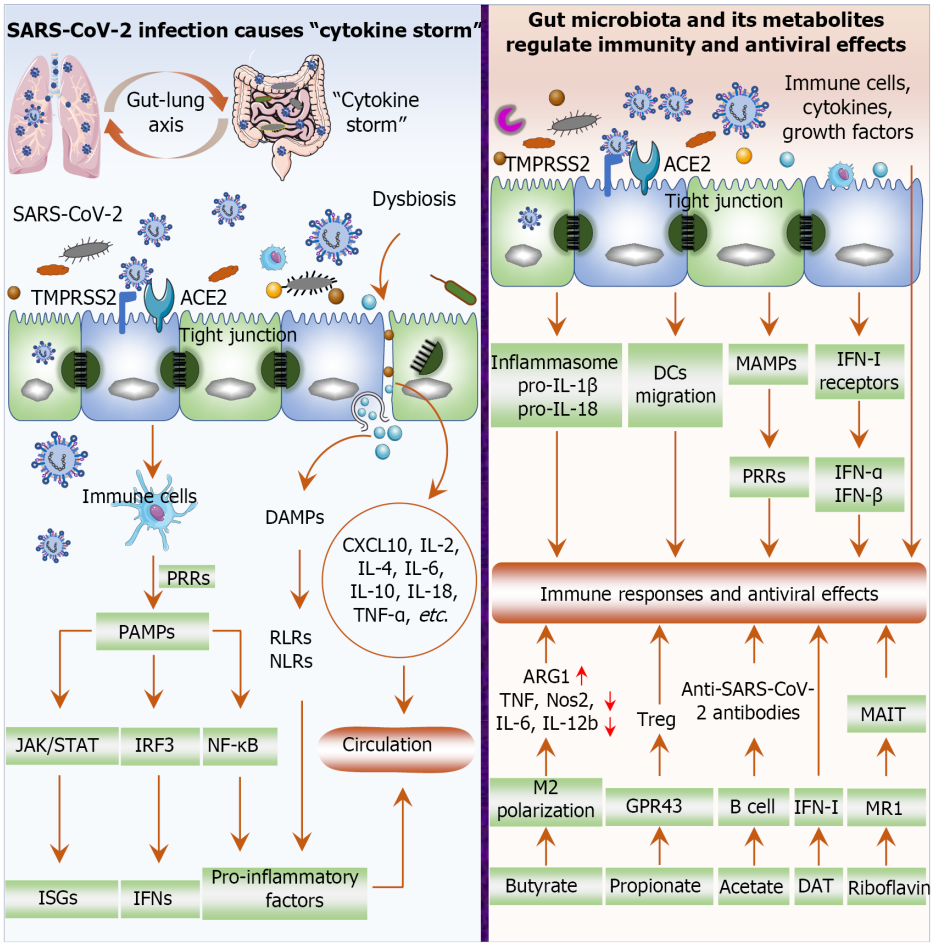

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Figure 2 Severe acute respiratory syndrome coronavirus 2 infection causes "cytokine storm" and the impact of gut microbiota and its metabolites on inflammatory response and antiviral effects. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) invades cells through angiotensin-converting enzyme 2 (ACE2) and transmembrane serine protease 2 (TMPRSS2) and replicates frantically, inducing innate immune cells [*e.g.*, macrophages, dendritic cells (DCs)] to recognize and bind pathogen-associated molecular patterns through pattern recognition receptors (PRRs). Subsequently, the expression of pro-inflammatory factors, interferons (IFNs) and IFN-stimulated genes is induced through the NF-κB, IRF3, and JAK/STAT signaling pathways, thereby promoting excessive inflammatory cytokine release. Killing or damaging cells by SARS-CoV-2 releases danger-associated molecular patterns that facilitate the expression of pro-inflammatory factors *via* activation of RIG-I-like receptors and NOD-like receptors. In addition, dysbiosis damages the gut barrier (tight junctions) and promotes the production of inflammatory factors such as CXCL10, IL-2, IL-4, IL-6, IL-10, IL-18, and TNF-α. Gut microbiota facilitates inflammasome activation, pro-IL-1β and pro-IL-18 expression, and DCs migration, thereby promoting protective immunity post viral infection. Microbial-associated molecular patterns are transmitted to the parenteral tissues to activate PRRs and affect innate immune responses. Gut microbiota regulates IFN-I receptors expression in respiratory epithelial cells and exerts antiviral effects through IFN-α and IFN-β. Immune cells, cytokines, and growth factors in the gut mucosa reach the respiratory tract to regulate immunity and exert antiviral effects. Butyrate promotes M2 macrophage polarization, upregulating arginase 1, and downregulating TNF, Nos2, IL-6, and IL-12b exerts anti-inflammatory activity. Propionate promotes Treg cell proliferation by activating G-protein-coupled receptor 43, thereby inhibiting autoinflammatory responses and protecting tissues from damage caused by pathological immune responses. Acetate promotes the production of SARS-CoV-2 antibodies by B cells, thereby inhibiting the development of COVID-19. The gut microbiota produces deaminotyrosine to enhance IFN-I signaling and protect the host from viral infection. Riboflavin, produced by the gut microbiome, activates mucosal-associated T cells *via* major histocompatibility complex-related protein-1. Mucosal-associated T cells participate in the immune response against SARS-CoV-2 through the gut-lung axis, thereby exerting antiviral efficacy. TMPRSS2: Transmembrane serine protease 2; ACE2: Angiotensin-converting enzyme 2; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; PRR: Pattern recognition receptors; IFN: Interferons; ISG: Interferons-stimulated gene; MAIT: Mucosal-associated T cells; MAMP: Microbial-associated molecular pattern; DAT: Deaminotyrosine; RLR: RIG-I-like receptors; NLR: NOD-like receptors; DAMP: Danger-associated molecular pattern; PAMP: Pathogen-associated molecular pattern; DC: Dendritic cell; MR1: Major histocompatibility complex-related protein-1; GPR43: G-protein-coupled receptor 43.

respiratory viral infections, which may be associated with dysregulated immune responses; increased secretion of IFN-γ, IL-6, and CCL2; and decreased Treg cell counts in the lung and gastrointestinal tract [59]. The gut microbiota facilitates inflammasome activation, pro-IL-1β and pro-IL-18 expression, and dendritic cell (DC) migration, which are critical for protective immunity post-influenza virus infection [60] (Figure 2). In addition, microbial-associated molecular patterns are transmitted to parenteral tissues to activate PRRs in immune cells and influence innate immune responses[61]. The gut microbiota reportedly regulates the expression of IFN-I receptors in respiratory epithelial cells that limit viral replication and increase resistance to viral infections through IFN-α and IFN-β[58,62]. The gut commensal microbiota promotes the IFN-I response and ISG expression by inducing IFN-β expression

mediated by TLR4-TRIF signaling through colonic lamina propria DCs, thereby enhancing antiviral capacity[62]. The gut microbiota can also affect respiratory mucosal immunity through multiple mechanisms. On the one hand, activated immune cells within the mucosa can reach and affect distant mucosal sites, highlighting the beneficial effects of the gut microbiota during respiratory viral infections [63,64]. On the other hand, the gut microbiota affects the secretion of cytokines and growth factors by the gastrointestinal mucosa, which reach the systemic circulation and act on other mucosal tissues[63, 65]. Furthermore, severe COVID-19 clinical symptoms or complications and higher mortality are more likely to arise in elderly individuals or in patients with concomitant underlying disease, such as cardiovascular disease, diabetes, and cancer[66]. Critically ill patients with COVID-19 often require invasive ventilator-assisted ventilation. However, gut microbiota intervention can reduce the demand for invasive mechanical ventilation in critically ill patients and reduce the incidence of ventilator-associated pneumonia[67], although this finding needs further validation in COVID-19 patients. Therefore, restoring gut microbiota homeostasis is essential for inhibiting the inflammatory response and enhancing antiviral effects.

Gut microbiota metabolites are signaling molecules and substrates for metabolic reactions[68]. Microbial metabolites are absorbed by the gut mucosa and participate in mucosal immune regulation, a process known as "metabolic reprogramming"[69,70]. For example, short-chain fatty acids (SCFAs, mainly including acetate, propionate, and butyrate) can reach distant organs through the bloodstream to exert immunomodulatory and immunoglobulin expression-inducing effects, as well as anti-inflammatory and antiviral effects[70,71] (Figure 2). Butyrate facilitates the polarization of M2 macrophage and exerts anti-inflammatory effects by increasing arginase 1 (ARG1) and decreasing TNF, Nos2, IL-6, and IL-12b expression[41,72]. Butyrate also inhibits histone deacetylase activity or increases the Foxp3 promoter transcription in naive T cells, thereby promoting the naive T cells differentiation into Treg cells[73,74]. Treg cells can suppress autoinflammatory responses and protect tissues from damage caused by pathological immune responses[41]. Furthermore, butyrate restored $CD8^+$ T-cell function in mice and inhibited proinflammatory cytokine production as well as eosinophilic lung infiltration[49]. Unfortunately, a reduced richness of butyrate-producing bacteria such as *F. prausnitzii* and *Clostridium* species has been found in COVID-19 patients[15,17,20,75]. Propionate promotes Treg cell proliferation by activating G-protein-coupled receptor 43 (GPR43)[76]. Moreover, microbe-produced SCFAs (acetate) enhance B-cell metabolism and gene expression, promoting the production of anti-SARS-CoV-2 antibodies and thereby inhibiting disease progression[77]. Deaminotyrosine (DAT) produced by *Clostridium orbiscindens* augments IFN-I signaling to protect the host from viral infection[57]. In addition, riboflavin is a product of gut microbiota constituents, which can activate mucosal-associated T cells (MAIT) *via* restrictive major histocompatibility complex (MHC)-related protein-1 (MR1)[78,79]. MAIT cells, as innate sensors and mediators of the antiviral response, exert antiviral response to SARS-CoV-2 through the gut-lung axis[80]. Antimicrobial peptides secreted by bacteria can promote virolysis, block cell virus fusion, and induce adaptive immune responses, and thus they have been proposed as viable alternative therapeutic treatments for infections by viruses, such as MERS-CoV[81]. Therefore, COVID-19 severity is tightly correlated with the levels of gut microbiota metabolites. However, studies on the direct action of gut microbiota metabolites in COVID-19 remain scarce.

REPROGRAMMING THE GUT MICROBIOTA AS A STRATEGY TO AMELIORATE GASTROINTESTINAL SYMPTOMS AND REDUCE DISEASE SEVERITY

The gut microbiota is closely related to various human gastrointestinal diseases and symptoms, such as gastrointestinal cancer, stomachache, diarrhea, and flatulence[82,83]. For instance, *Escherichia coli*, *Shigella*, *Salmonella*, *Campylobacter*, *Clostridium difficile*, and *Aeromonas* are the main pathogens that cause diarrhea[84,85]. Reprogramming the gut microbiota *via* probiotics, prebiotics and FMT is well documented to effectively treat gastrointestinal diseases. Moreover, probiotic/prebiotic administration has been shown to protect against viral infections in multiple studies (*e.g.*, those caused by influenza virus, rhinovirus, respiratory syncytial virus and coronaviruses). For example, Johnson *et al*[86] found that *Bacillus subtilis* peptidoglycans reduced the infectivity of coronavirus. Peptidoglycan-associated surfactin can disrupt virion integrity, such as in influenza, Ebola, Zika, and Mayaro[86]. Therefore, probiotics, prebiotics and FMT can restore ecological homeostasis by regulating the gut microbiota, representing an effective alternative approach to ameliorate or suppress COVID-19 severity.

Probiotics, such as *Lactobacillus*, yeast, *Bifidobacterium*, *Enterococcus*, and *Bacillus*, are live microorganisms that benefit the host by colonizing the human body. Probiotics maintain healthy gut homeostasis and exert antiviral effects through the gut-lung axis[87,88] and have been widely used to treat gastrointestinal diseases, including diarrhea (acute, antibiotic-associated and *C. difficile*-associated) and adult inflammatory bowel disease (IBD)[89-91]. On the one hand, probiotics can strengthen Treg cell and natural killer cell activity and suppress proinflammatory cytokines, such as TNF-a, CRP, IL-1b, IL2, IL-6, IL7, MCP1, and LDH[92,93]. *Lactobacillus* exerts antiviral activity through direct probiotic-virus interactions, production of antiviral metabolites, and stimulation of the immune system[94]. On the other hand, probiotics are beneficial for enhancing epithelial barrier function and improving gut

microbial diversity. Furthermore, probiotics combat and block harmful bacterial strains in the gut or enhance beneficial signaling pathways[95,96]. Existing evidence shows that probiotic miRNA modulation and regulation of signaling pathways such as NF-kB and STAT1 ameliorate COVID-19 complications[97,98]. Probiotics have the potential to interact with ACE2; for example, some lactobacilli release peptides with a high affinity for ACE2[93,99]. *Lactobacillus spp.* and *Bifidobacterium spp.* exhibit the strongest anti-respiratory virus activity *via* an immunomodulatory mechanism[56]. In January 2020, relevant Chinese government departments recommended the addition of probiotics in COVID-19 treatment to improve gut microbiota homeostasis and protect against subsequent bacterial infections. However, evidence detailing the efficacy of probiotics in treating COVID-19 is limited, although relevant clinical trials are being conducted. Moreover, the safety of probiotics in COVID-19 patients should be emphasized to ensure that their use does not induce new gastrointestinal symptoms or secondary infections.

Prebiotics are substrates that are selectively utilized by host microorganisms to provide health benefits[100]. Unfortunately, little information is available on using prebiotics to treat respiratory infections. In a clinical trial of prebiotics, KB109 was found to regulate gut microbiome composition and increase the production of SCFAs in the gut (NCT04414124). For instance, butyrate and propionate obtained from the fermentation of prebiotics affect the differentiation or function of T cells, macrophages, and DCs[93]. In addition, prebiotics selectively stimulate beneficial bacterial growth or enhance the activity of indigenous probiotics[93]. Accordingly, the role of prebiotics in COVID-19 patients should be further emphasized.

FMT refers to the transplantation of a functional microbiota from healthy individuals into the gastrointestinal tract of patients to treat intestinal and extraintestinal diseases[84]. An expanding body of evidence suggests that FMT can effectively treat many diseases, such as cancer, liver diseases, *C. difficile* infection, irritable bowel syndrome (IBS), and IBD[101-103]. Since COVID-19 patients exhibit a depletion of beneficial commensal bacteria, FMT represents a theoretically promising strategy to mitigate disease severity. Regrettably, convincing clinical evidence for the effect of FMT in COVID-19 is lacking, and its safety and efficacy warrant further investigation.

CONCLUSION

COVID-19 was previously identified as a respiratory infectious disease. However, accumulating clinical studies have subsequently found that a large proportion of patients have gastrointestinal symptoms, such as abdominal pain, diarrhea, vomiting, and acid reflux, as well as significant gut microbiota alterations[7,30]. The gut microbiota not only significantly affects the COVID-19 development and disease severity but also reflects the susceptibility of COVID-19 patients to long-term complications [104]. Studies have confirmed that dysbiosis increases the poor prognosis of COVID-19[104]. However, controversial results exist regarding gut microbiota alterations in COVID-19 patients. A variety of factors, such as sex, age, basic health status, medication use, genetics, ethnicity, and geographic location, can affect the composition of the gut microbiota and lead to individual differences and varying responses to SARS-CoV-2 infection. Therefore, further exploration of the specific gut microbiota alterations in COVID-19 patients and the clinical correlation between the gut microbiota and COVID-19 will be a very challenging and valuable research direction in the future.

Unfortunately, direct evidence for the contribution of the gut microbiota in COVID-19 remains lacking. For example, the exact mechanism of dysbiosis remains unclear. Moreover, the regulation of host immune and inflammatory responses and antiviral effects by the gut microbiota during SARS-CoV-2 infection relies largely on inferences or conjectures from previous studies. Given that specific drugs for COVID-19 remain enigmatic, vaccines represent the most effective prevention and control strategy; however, the continuous mutation of the virus has exacerbated this conundrum. Therefore, gut microbiota intervention through probiotics, prebiotics and FMT is undoubtedly one of the promising cosupplementation strategies for the future treatment of COVID-19, but large-scale studies are lacking and there are no corresponding treatment guidelines. The therapeutic prospects of the gut microbiota in COVID-19 are promising, but there is still a long way to go to realize its potential.

FOOTNOTES

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MINIREVIEWS

Microbiota in the stomach and application of probiotics to gastroduodenal diseases

Yasuhiro Koga

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Abstract

The stomach is a hostile environment for most microbes because strong gastric acid kills indigenous microorganisms. Thus, the mass of indigenous microbes detected by traditional culturing method in a highly acidic stomach is reported to be very small. However, in a stomach with less acidity due to atrophic changes of the gastric mucosa, the number of live gastric microbiota dramatically increases and their composition changes. A probiotic is defined as a live microorganism that, when administered in adequate amounts, confers a health benefit on the host. The administration of probiotics to the stomach has thus far been considered impractical, mainly due to the strong acidity in the stomach. The identification of candidate probiotic strains with sufficient resistance to acidity and the ability to achieve close proximity to the gastric mucosa could enable the application of probiotics to the stomach. The utilization of probiotics alone for *Helicobacter pylori* (*H. pylori*) infection significantly improves gastric mucosal inflammation and decreases the density of *H. pylori* on the mucosa, although complete eradication of *H. pylori* has not yet been demonstrated. The use of probiotics in combination with antimicrobial agents significantly increases the *H. pylori* eradication rate, especially when the *H. pylori* strains are resistant to antimicrobial agents. While *H. pylori* has been considered the most important pathogenic bacterium for the development of gastric cancer, bacteria other than *H. pylori* are also suggested to be causative pathogens that promote the development of gastric cancer, even after the eradication of *H. pylori*. Increased non-*H. pylori* Gram-negative bacteria in the stomach with weak acidity accompanying atrophic gastritis may perpetuate gastric mucosal inflammation and accelerate carcinogenic progression, even after *H. pylori* eradication. Probiotics restore the acidity in this stomach environment and may therefore prevent the development of gastric cancer by termination of Gram-negative bacteria-induced inflammation. Functional dyspepsia (FD) is defined as the presence of symptoms that are thought to originate in the gastroduodenal region in the absence of any organic, systematic or metabolic diseases. Accumulating evidence has pointed out the duodenum as a target region underlying the pathophysiology of FD. A randomized placebo-controlled clinical trial using a probiotic strain (LG21) demonstrated a significant improving effect on major FD symptoms. One of the possible mechanisms of this effect is

protection of the duodenal mucosa from injurious intestinal bacteria through the resolution of small intestinal bacterial over growth.

Key Words: Stomach; Microbiota; Probiotics; *Helicobacter pylori*; Post-eradication gastric cancers; Functional dyspepsia

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Core Tip: Gastric microbiota and application of probiotics to the gastroduodenal diseases have so far been unfamiliar because the mass of live microbes is so small in the stomach with high acidity. However, in the subject whose stomach is low acidity due to atrophic gastritis or proton pump inhibitor long-use, the number of live bacteria increases so much in the stomach thus they can significantly influence the pathophysiology of gastroduodenal diseases.

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INTRODUCTION

The intestine is colonized by a complex and dynamic microbial ecosystem with a high density of bacteria whose cell number can reach as high as approximately 10^{12}/g feces. Their total number is therefore estimated to be 10-times greater than the number of eukaryotic cells in the human body, and the genes of these microbes outnumber human genes more than 100-fold[1]. As the early definition of probiotics emphasized their effects on improving the ecology of intestinal microbiota, their actions on the intestinal tract and gut mucosal immunity have received a great deal of attention.

On the other hand, the size of gastric microbiota, in which probiotic bacteria exert their beneficial effects, is very small [around 10^3 colony-forming units (CFU)/mL gastric fluid (GF)[2]] because of the strong acidity and frequent peristalsis to the intestine in the stomach. Such high acidity due to secreted gastric acid kills probiotic strains as well as gastric commensal microbes. Therefore, the application of probiotics to the stomach or proximal small intestine has historically been considered impractical.

Based on the outline of microbiota and probiotics in the stomach, this article reviews *Helicobacter pylori* (*H. pylori*) infection and functional dyspepsia (FD). The former includes the pathogenicity of *H. pylori*, the suppressive mechanism of probiotics on this bacterium and the present status of the application of probiotics in eradication therapy. In addition, this review argues a possible role of probiotics in the prevention of post-*H. pylori* eradication gastric cancer. In the description, basic and clinical data reported by the author's group are emphasized because they are indispensable for communicating the author's ideas in relation to the theme of this review article.

MICROBIOTA IN THE STOMACH

Probiotics are considered to exert beneficial effects on the host by improving the indigenous microbiota [3]. Thus, a brief description of the gastric microbiota is necessary to understand the effects of probiotics in the stomach. The stomach is a hostile environment for most microbes because strong gastric acid kills indigenous microorganisms. Therefore, when examined using traditional culturing methods, bacterial numbers in the gastric mucosa-associated or GF are reported to contain only approximately 10^3 CFU per g or mL[2]. Moreover, this method can only detect microbes that are able to grow in the media components and the atmospheric conditions of the culturing assay. Thus, in the stomach-where the acidity is high enough to kill indigenous bacteria-the investigation of gastric microbiota by traditional culturing methods makes no sense as the stomach contains few live bacteria. However, the introduction of DNA sequencing using high performance next-generation sequencers has markedly enhanced the analysis of microbiomes in the stomach as well as in the intestine. In a stomach with less acidity due to the continuous use of acid-suppressive agents [*e.g.*, proton-pump inhibitors (PPIs)] or atrophic change of the gastric mucosa, the number of bacteria in the live gastric bacterial mass dramatically increases and the composition changes, respectively[4,5]. In these low acidity settings, the gastric microbiota will exert a significant effect on the pathophysiology of upper gastrointestinal (GI) tract disorders.

The mouth is located at the entrance of the GI tract, and contains complex anatomical sites, including the teeth, gingiva and tongue. The oral microbiota flows downstream into the stomach by swallowing of saliva and mastication of foods. These are expected to exert a great influence on the microbiota in the stomach. While the gut harbors a very large and complex microbial community, it is conceivable that such intestinal microbiota can be significantly affected by the gastric microbiota through continuous inflow. This is especially true after a large increase in the bacterial mass in a stomach with low acidity. This raises the hypothesis that the GI tract has a common microbial ecosystem in which the gastric microbiota plays the role of a relay base between the oral and gut microbiotas. Therefore, in this chapter, the microbiota in the stomach is described and compared to the microbiotas of the oral cavity and gut. *H. pylori* is not an indigenous resident but a pathogen of exogenous origin. Accordingly, it is not described in the present chapter.

In 2006, Bik *et al*[6] identified 128 bacterial phenotypes based on a 16S rRNA gene analysis of gastric microbiomes with 1833 sequences obtained from 23 human gastric endoscopic biopsy specimens. A few years later, Li *et al*[7] also performed a 16S rRNA gene analysis using 1223 non-*H. pylori* sequences of 10 biopsy samples from the stomach, which were then classified into 133 phylotypes. Despite examining racially distinct populations (North America and China, respectively), both studies investigating the gastric mucosa-associated microbiomes revealed similar results. *Streptococcus* and *Prevotella* were the predominant genera, and they accounted for approximately half of the total detected species detected in their studies. In 2015, Tsuda *et al*[2] performed a meta-16S analysis of the gastric luminal microbiome with far greater sequencing depth. Their analysis was performed using GF samples obtained from Japanese subjects in a fasting state in the morning. They obtained roughly 40000 high-quality reads for the analysis from 45 GF specimens and also identified *Streptococcus* and *Prevotella* as the most prevalent genera, accounting for approximately 50% of all the species detected in the stomach (Figure 1). Moreover, in all three of these studies, *Neisseria* and *Rothia* ranked among the top 5 most prevalent genera. This similarity in the bacterial composition between the mucosa-associated[6,7] and luminal[2] samples suggested that the former bacteria moved back into the lumen, while the latter continuously colonized the mucosa. *H. pylori* is a predominant inhabitant of the mucous layer and also inhabits the gastric epithelial cells. Accordingly, it is mainly found in the mucosa-associated specimens.

Tsuda *et al*[2] also compared three different bacterial communities along the alimentary tract (oral cavity, stomach and colon) using stimulated saliva, GF, and feces specimens. There was no significant difference in the degree of species richness (α-diversity) among the three types of specimens. While the median log CFU bacterial number was only 3.4/mL (determined by culturing), the bacterial log genome copy number was as high as 7.8/mL (median) in GF samples. This large discrepancy between the CFU and genome copy numbers implied that > 99.9% of the GF bacteria were dead or viable but non-culturable. The analysis of bacterial genome copies also suggested that-if microbes were alive and metabolically active in the stomach with weak acidity-the mass size of the GF microbiota may be high enough to significantly affect the pathophysiology of the stomach and its downstream organs. A bacterial composition analysis at the genus level showed high similarity between the salivary and GF microbiota (Figure 1). Indeed, the five most prevalent genera in these microbiomes (in descending order) were as follows: *Streptococcus, Prevotella, Neisseria, Rothia* and *Veillonella,* and *Streptococcus Prevotella, Actinomyces, Neisseria* and *Rothia*. In contrast, the composition of the fecal microbiota was markedly different.

In a meta-16S analysis, which was performed to investigate the influence of gastric acidity on the microbiome composition in the stomach without atrophic change of the mucosa, PPI treatment was found to significantly increase the amount of *Streptococcus* in the mucosa-associated gastric microbiota [8]. This increase occurred independently of *H. pylori* infection. The compositions of other bacteria at the genus level showed no significant alteration. In another study, in which a bacterial DNA analysis study was conducted using GF samples, PPI-treated subjects whose GF acidity was > pH 4, showed lower α-diversity than subjects without PPI treatment[9]. No other significant changes in the GF microbiome composition were observed in GF samples with weak acidity. Furthermore, the presence of *H. pylori* was not associated with the difference in the microbiome composition. Moreover, in a study of PPI-users by Tsuda *et al*[2], no significant changes were observed in the bacterial composition (at the genus level) in the GF of PPI users, with the exception that *Streptococcus* tended to be more prevalent (Figure 1). In their study, the average pH values of GF samples obtained from the PPI-users and PPI-nonusers were 3.2 and 1.6, respectively. Of note, the average log copy number of bacterial cells (/mL) in these GF samples (measured by quantitative polymerase chain reaction) was almost the same in PPI-users (8.0) and PPI-nonusers (8.1), while the CFU number (determined by culturing) was > 1000-fold greater in PPI-users with weak acidity. This result implies that PPI-induced low acidity protected the gastric microbiota from strong acid.

Bacterial overgrowth in the stomach with weak acidity has been suggested to occur due to the restoration of active growth of relatively acid-resistant indigenous bacteria, which are kept alive (in small numbers) due to their suppressed growth in the strongly acidic stomach. However, the high similarity in the bacterial community structure between the GF and saliva, and the high similarity of their bacterial genome copy numbers, suggests that no "bacterial overgrowth" occurred. Instead, the bacteria that moved from the oral cavity to the stomach with weak acidity simply avoided being killed by gastric acid. Although PPI use was not associated with any significant alteration in the composition

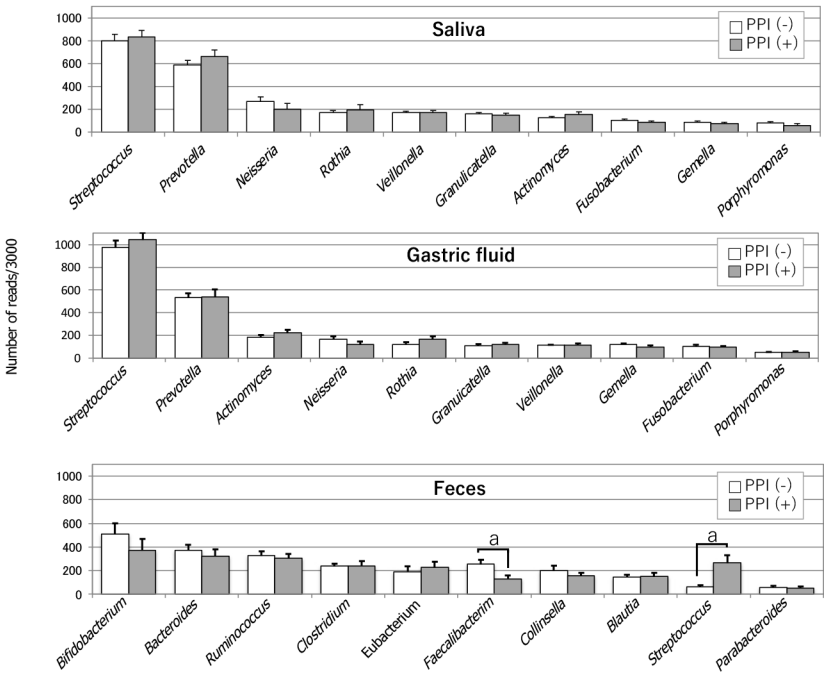

Figure 1 Comparison of the microbiota in the saliva, gastric fluid and feces, and the influence of proton-pump inhibitors. Bacterial compositions at the genus level in saliva (top), gastric fluid (median) and feces (bottom) are shown. The average of read numbers of the top 10 major genera are indicated in each group. Open and filled bars represent proton-pump inhibitor (PPI)-nonusers and PPI-users, respectively. Asterisks show a significant difference according to Student *t*-test. $^{a}P < 0.05$. PPI: Proton-pump inhibitor. Citation: Tsuda A, Suda W, Morita H, Takanashi K, Takagi A, Koga Y, Hattori M. Influence of Proton-Pump Inhibitors on the Luminal Microbiota in the Gastrointestinal Tract. *Clin Transl Gastroenterol* 2015; 6: e89. Copyright ©Wolters Kluwer Health, Inc. 2015. Published by Wolters Kluwer Health, Inc.

of the gastric microbiota, a significant decrease of *Faecalibacterium* and a significant increase of *Streptococcus* were found in the feces of PPI-users (Figure 1). A reduction in the abundance of *Faecalibacterium* was also reported in the feces of dogs treated with PPIs[10]. Whether the large increase of live bacteria induced by PPI treatment significantly influences the intestinal bacterial community structure remains to be elucidated.

PROBIOTICS FOR THE STOMACH

In 1989, Fuller defined probiotics as "a live microbial feed supplement that beneficially affects the host animals by improving its intestinal microbial balance"[3]. This early and influential definition was refined by a standard definition proposed by the Joint (Food and Agricultural Organizations of the United Nations)/World Health Organization Expert Consultation in 2001[11]: "a live microorganism that, when administered in adequate amounts, confers a health benefit on the host". The International Scientific Association for Probiotics and Prebiotics consensus statements reported in 2014 exclusively retained the body of these definitions[12].

The main beneficial effects of probiotics on the host include inhibition of potential pathogens in the GI tract, modulation of immunity, and reinforcement of the mucosal barrier. Competition with bacteria for binding sites on the mucosa by probiotic strains is the dominant mechanisms underlying the protection of the host from pathogenic bacteria in the intestine. Organic acids secreted by probiotic strains (*e.g.*, lactic acid) and short-chain fatty acids (*e.g.*, acetic acid) are considered to exert a significant bactericidal effect on the pathogens. As underlined in the definitions of probiotics, a "living" state is indispensable for probiotics to protect the hosts from pathogens, because dead probiotic strains no longer have the ability to specifically bind to the mucosa or generate organic acids.

Because the early definition of probiotics emphasized their beneficial effects on improving intestinal microbial ecology, it has been considered that probiotics should be exclusively applied to the gut. Actually, the gut is colonized with a high density of bacteria, the cell number of which can reach as high as 10^{12}/g feces[13]. In contrast, the size of bacterial mass in the stomach, in which probiotic strains could work, is small. The number of indigenous bacteria in the gastric fluid of healthy stomach-when examined by culturing methods- is at most 10^{3}/mL[2,4]. The strong acidity in the stomach due to secreted gastric acid, which causes a marked reduction in the size of such resident gastric bacteria also suppresses or terminates the beneficial effects exerted by probiotic strains soon after they move to the stomach. For this reason, the application of probiotics to the stomach or proximal small intestine has so

far been considered impractical.

The proximity of probiotic strains to the mucosa-which will be achieved by bearing affinity to the surface mucus layer, or by adhering to the epithelial cell layers-is crucial for beneficial effects to be exerted in the GI tract[14]. Those effects include competitive inhibition of the pathogenic bacteria's attachment to the mucosa, secretion of organic acids in effective concentrations, and contact-dependent immunomodulation. Peristaltic movements in the stomach are more frequent and active in comparison to the intestine; thus, it is difficult for probiotic strains to come into close proximity to the gastric mucosa. This factor also makes the use of gastric probiotics difficult.

From another point of view, the small size of resident microbiota in the stomach can easily be affected by the introduction of exogenous microorganisms. These transient bacteria (*e.g.*, ingredients of foods or accidental contaminants) exert a much greater effect on the microbiota in the upper GI tract than on the gut microbiota due to its much smaller size. The same situation is encountered with the administration of probiotics to the upper GI tract, where they are not disturbed by large amounts of robust and resilient microbiota, which are present in the gut. The application of probiotics to the upper GI tract may therefore be more advantageous in comparison to application to the gut, as long as the candidate strains are able to resist the strong acidity and achieve close proximity to the gastric mucosa.

The stomach is a harsh environment for most microorganisms because strong gastric acid kills many ingested microbes. In a fasting state in the morning, GF has a peak acidity of pH 1-2. Such strong acidity is quite reasonable because one major physiological role of gastric acid is to kill exogenous pathogenic bacteria that move to the digestive tract through the mouth. At present, the most prevalent probiotic strains belong to genera *Lactobacillus* or *Bifidobacterium*[11]. In general, *Lactobacilli* and *Bifidobacteria* show considerably high resistance to acidity. While both bacterial groups can survive acidic conditions of approximately pH 3 to 4[15], this is not as strong as the peak gastric acidity (pH 1-2). Thus, screening using candidate probiotic strains is necessary to identify a suitable probiotic strain for the stomach that can tolerate approximately pH 2.

Genus *Lactobacillus* is one of the predominant resident bacterial groups found in the stomach (when examined by culturing methods)[16]. Accordingly, *Lactobacillus* strains might be the most appropriate for use as probiotics in the stomach. Furthermore, in adult mice with a specific pathogen-free (SPF) environment, no *H. pylori* infection was found in the stomach after oral inoculation of the bacteria[16]. In contrast, mice bred in a germ-free environment were easily infected by oral inoculation of *H. pylori*. In this animal study, *H. pylori* infection was prevented in SPF mice with a large number of indigenous lactobacilli in the stomach (> 10^8 CFU/g tissue). A representative probiotic strain that can be used for the stomach is *Lactobacillus gasseri* OLL2716 (LG21). This was selected out of approximately 2000 strains of lactobacilli[17]. The criteria for selection were both resistance to acidity and the ability to bind to the gastric mucosa. In the stationary growth phase, LG21 can survive in culture broth at pH 2.5, which is similar to the acidity of GF. LG21 has several defense mechanisms that enable it to withstand acid stress, including the up-regulation of the cation ATP-binding cassette transporter genes and the downregulation of the genes associated with transcription and protein synthesis[18]. The acid stress response is generally indispensable for lactobacilli, because they always secrete large amounts of organic acids to the external environment when they grow with metabolic activity. Without this defense mechanism, the acidic milieu induces the arrest of the growth of lactobacilli and it may even cause their death.

In a handful of trials, endoscopy directly demonstrated mucosal colonization by probiotics. Using biopsy samples obtained by upper GI endoscopy, LG21 strains administered through a yogurt drink were shown to be able to enter the mucous layer of the human stomach[19]. The laser-assisted microdissection and non-contact pressure catapulting method enabled this fine topical analysis.

H. PYLORI INFECTION

H. pylori and its pathogenicity

H. pylori is a gram-negative and microaerophilic bacterium that can move in the mucus layer on the surface epithelial cells of the stomach using several of flagella that are located at one end (Figure 2). As much as half of the people in the world are infected with *H. pylori*[20]. *H. pylori* infection causes inflammation of the gastric mucosa and then leads to a gradual loss of hydrochloric acid-secreting parietal cells of the stomach. This ultimately results in a condition known as atrophic gastritis. Atrophic gastritis is a chronic inflammatory and low gastric acidity state that has a high risk of progressing to gastric cancer[21].

H. pylori can tightly bind to epithelial cells by multiple bacterial-surface components. The best-characterized adhesin, BabA, is a 78 kD outer-membrane protein that binds to the fucosylated Lewis B blood group antigen on the host cell[22]. Firm contact between *H. pylori* and the host cell through adhesion is considered a prerequisite for the *H. pylori* to transport effecter molecules (*e.g.*, CagA) into the host cell using the *cag* PAI-encoded type IV secretion system[23]. This event is regarded as the pathway leading to the generation of proinflammatory cytokines (*e.g.*, IL-8 and IL-1β) by host cells. Thus, the adhesion of *H. pylori* to epithelial cells is a critical event in the development of an inflammatory response and the establishment of infection in the stomach.

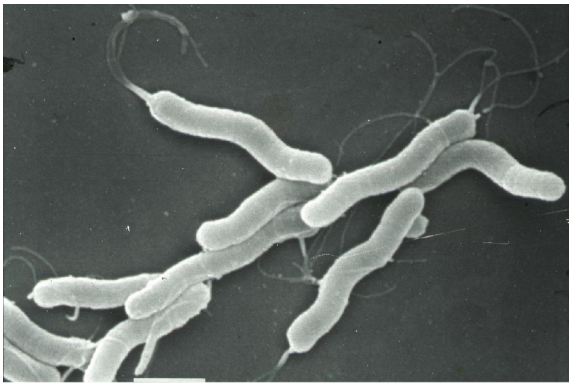

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Figure 2 Observation of *Helicobacter pylori* by scanning electron microscopy. The bar at the bottom shows 1 μm.

H. pylori eradication

Mechanism of the suppressive effect of probiotics on *H. pylori*: The major mechanisms of probiotics against *H. pylori* infection are thought to be competition with *H. pylori* for binding sites on gastric epithelial cells, reinforcement of the mucosal barrier, and secretion of bactericidal organic acids (*e.g.*, lactic acid). These are the principle anti-bacterial effects exerted by probiotics. As for the mechanism of competitive binding, *L. reuteri* is reported to inhibit the attachment of *H. pylori* on the epithelial cell surface by competitive binding to asialo-GM1 and surface receptors[24]. Moreover, other probiotic species (*e.g.*, *L. acidophilus*, *L. johnsonii* and *L. salivarius*) were reported to prevent *H. pylori* colonization through specific adhesion molecules[25,26]. Specific binding of *H. pylori* to the host cell then induces the production of IL-8 through the type IV secretion system. Tamura *et al*[27] also demonstrated competition for binding sites between *H. pylori* and a probiotic strain LG21 using a coculture system with MKN45 cells (a human gastric epithelial cell line) and *H. pylori*. Large amounts of IL-8 were produced in the gastric epithelial cells cocultured with *H. pylori* (10^6 CFU) alone. When 10^6 CFU of non-treated live LG21 (equivalent to the number of *H. pylori*) was added to the coculture system, the amount of IL-8 secreted into the culture supernatant significantly decreased. However, UV- or heat-treated LG21 could not exert any suppressive effect on *H. pylori*-induced IL-8 production, even at 10^8 CFU (100 times the amount of non-treated LG21). An adherence assay in their study supported that LG21 competitively inhibited the binding of *H. pylori* to MKN45 cells, which suppressed the production of IL-8. Moreover, they demonstrated that the suppressive effect of LG21 also worked in the human stomach. The measurement of the IL-8 Level in gastric biopsy specimens from *H. pylori*-infected subjects also revealed that the oral intake of probiotic LG21 significantly suppressed the generation of IL-8 in the gastric mucosa[27].

Application of probiotics in the eradication therapy: The clinical application of probiotics in the treatment of *H. pylori* infection has been performed in many countries for more than 20 years. Now the utilization of probiotics alone for *H. pylori* infection has almost been settled. Both early and recent reviews[28,29] concluded that probiotics significantly improved gastric mucosal inflammation, and decreased the density of *H. pylori* on the mucosa. However, to our knowledge, the complete eradication of *H. pylori* colonizing the stomach by probiotic treatment alone has not been demonstrated. One representative trial of probiotics alone for the treatment of *H. pylori* infection was reported by Sakamoto *et al*[17] in 2001. In their study, 31 *H. pylori*-infected subjects (mean age 50 years) ingested yogurt containing 10^9 CFU of LG21 or placebo yogurt without LG21 every day for 8 wk. The results of ^{13}C-urea breath tests (UBT) and assays of serum pepsinogens I and II (PGI/II) showed a significant clinical improvement after LG21 yogurt treatment. The ^{13}C-UBT result and the PGI/II ratio are known to indirectly represent *H. pylori* density and the degree of mucosal inflammation in the stomach, respectively[30,31]. A bacterial examination of gastric mucosal biopsy specimens (by culturing) revealed 2-100-fold decreases in the number of *H. pylori*. However, there were no subjects in whom *H. pylori* was completely eliminated. Pantoflickova *et al*[32] reported the effects of the administration of *L. johnsonii* La1 (LC-1) to 50 *H. pylori*-positive healthy volunteers in a randomized controlled, double-blind study. The subjects received 125 g of fermented milk containing 10^6-10^7 CFU/g of LC-1 or placebo milk without LC-1 every day for 16 wk. The severity/activity of antral gastritis (assessed histologically) and the *H. pylori* density (assesses by a ^{13}C-UBT) showed significant improvement. The histological examination of the mucous mucosa also revealed a significant increase in the mucous thickness in the LC-1-treated group. This suggested that the stabilization of the mucosal barrier by probiotics also enhanced the suppression of *H. pylori*.

Recently, the *H. pylori* eradication rate in patients treated using anti-microbial agents is decreasing. This is mainly due to antimicrobial resistance. In the early 1990s, the standard triple therapy achieved an eradication rate of > 90%. In contrast in the past decade, the effectiveness of this regimen often falls to < 70%[33,34]. According to an ITT analysis by Deguchi *et al*[35] in 2012, the successful eradication rate

using the same regimen was just 69.3%. In those subjects, the rate of infection with clarithromycin-resistant strains of *H. pylori* was as high as 27.1%. This increase in resistance to antimicrobials like clarithromycin is thought to have reduced the eradication rate. Actually, the clarithromycin resistance rates of *H. pylori* isolated from children in North America and Europe were reported to be 10.6%-25% and 1.7%-23.4% respectively[36,37]. These studies also reported the increasing prevalence of *H. pylori* isolates that are resistant to metronidazole, which is frequently used in the first-line regimen.

The use of probiotics in combination with antimicrobial agents significantly increased the eradication rate, especially for bacteria with antimicrobial resistance. Both the suppressive effect on *H. pylori* by probiotics and the compliance-promoting effect of ameliorating the side effects of antimicrobials are thought to significantly increase the eradication rate. Actually, Deguchi *et al*[35] reported that a group treated with one week-triple therapy supplemented with LG21 yogurt and a group with triple therapy alone showed cure rates of 82.6% and 69.3%, respectively. The difference in the intention-to-treat analysis was statistically significant ($P = 0.018$). In their study, 112 g of yogurt containing 10^9 CFU of LG21 was given twice daily for 4 wk (3 wk of pretreatment and 1 wk during eradication therapy). According to a recent meta-analysis of 40 eligible studies with 8924 patients[29], the use of probiotics before and throughout the eradication treatment was associated with a superior eradication effect. Patients who received supplementary probiotics showed a higher eradication rate [relative risk (RR) 1.14, 95%CI: 1.10-1.18, $P < 0.001$] and lower incidence of total side effects (RR 0.47, 95%CI: 0.39-0.57, $P < 0.001$) in comparison to the control group without probiotics. In a sub-analysis, *Lactobacillus* was the best choice among the probiotic strains, and probiotics combined with bismuth quadruple regimen was suggested to be the best combination.

Possible role of probiotics in preventing post-eradication gastric cancer: The lifetime risk of gastric cancers in *H. pylori*-infected individuals is estimated to be 3%-5%[38]. In *H. pylori*-infected patients, colonization with *H. pylori* on the gastric mucosa is known to gradually decrease overtime and often becomes undetectable in patients who develop gastric cancer[39]. Furthermore, during long-term follow-up (up to approximately 20 years) of patients who had been cured of *H. pylori* infection at the start of observation, 0.35% of subjects developed gastric cancer per year. That is, 7% is estimated to have developed gastric cancer at 20 years[40]. These findings strongly suggest that there are some causative factors other than *H. pylori* can also promote the development of gastric cancer even after *H. pylori* eradication. However, *H. pylori* is currently considered the most important pathogen for the development of gastric cancer.

According to the Correa pathway[41], chronic *H. pylori* infection progresses over the decades through the following stages: chronic gastritis, atrophy, intestinal metaplasia, and cancer. Gastric adenocarcinomas are classified as both well-differentiated (intestinal-type) and undifferentiated (diffuse-type) ones[42]. The development of gastric atrophy is recognized as a critical step to the development of intestinal-type gastric cancer in the Correa pathway. Mucosal atrophy is usually accompanied by inflammation, and is thus recognized as atrophic gastritis. Accordingly, atrophic gastritis appears to be the strongest risk factor for gastric cancer[21]. The histological characteristics of the gastric mucosa (*e.g.*, inflammation, atrophy, and intestinal metaplasia) were analyzed to identify risk factors for gastric cancer after *H. pylori* eradication[43]. The mucosal inflammation score of the group who developed gastric cancer after successful *H. pylori* eradication ($n = 61$) was significantly higher than the group without cancer after eradication ($n = 122$). The RR and 95%CI were 5.92 and 2.11-16.6, respectively ($P < 0.01$). Neither atrophy nor intestinal metaplasia itself was a direct risk factor for post-eradication cancer.

The gastric corpus and antrum predominantly contain acid-secreting parietal cells and gastrin-secreting G cells, respectively. Thus, the mucosal atrophy in the corpus caused by *H. pylori* infection rapidly leads to a reduction in the gastric acid production. In contrast, the production of gastrin (an acid secretion stimulating hormone) remains relatively unchanged. Of note, patients who develop *H. pylori*-associated duodenal ulcers seem to be somewhat protected from the occurrence of gastric cancers[44]. The predominant mechanism of this protection in patients with duodenal ulcer from the cancers appears to be a higher basal level of gastric acid secretion. On the contrary, it was reported that the long-term suppression of gastric acid secretion by proton pump inhibitors (PPIs) was associated with a significantly increased risk of gastric cancer in *H. pylori*-infected subjects[45]. During approximately 8 years of follow-up, Cheung *et al*[46] evaluated the gastric cancer risk in patients treated with PPI using a Cox proportional hazards model. The study population consisted of approximately 63000 subjects, who had received clarithromycin-based triple therapy for *H. pylori* eradication. The use of PPIs was associated with an increased gastric cancer risk (HR 2.44, 95%CI: 1.42-4.20). This result demonstrated that the long-term use of PPIs was still associated with an increased risk of gastric cancer, even after *H. pylori* eradication. Accordingly, the stomach with low acidity accompanied by gastritis with atrophy and/or intestinal metaplasia must be considered a high-risk environment that predisposes the gastric mucosa to the development of gastric cancer.

Gastric acid reduction invariably results in a marked increase in the number of non-*H. pylori* bacteria. Due to the low acidity, these bacteria are still viable and show metabolic activity in the stomach. It therefore appears likely that such an enlarged bacterial mass causes the development of gastric cancers even after *H. pylori* eradication. Recent studies on the characteristics of the gastric microbial changes associated with gastric carcinogenesis revealed a reduction of species richness, the enrichment of

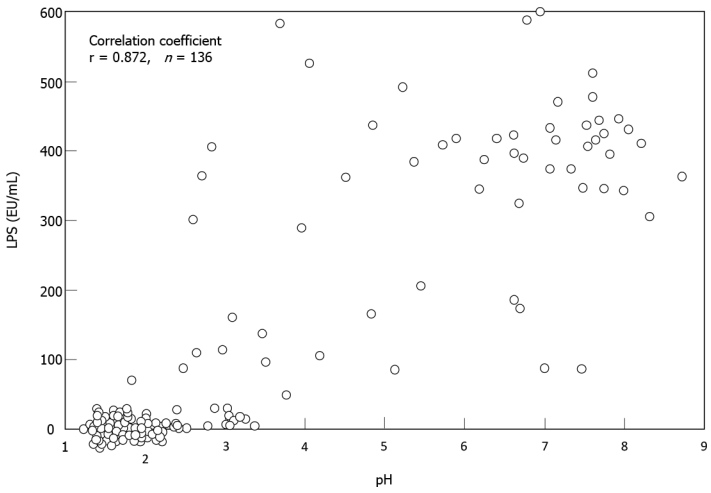

Figure 3 Correlation between pH and lipopolysaccharide activity in gastric fluid. The pH values and lipopolysaccharide activity of gastric fluid samples from 136 subjects were examined using a recombinant factor C assay kit. The correlation coefficients of the both parameters by Spearman test (*r*) is shown on the upper left. LPS: Lipopolysaccharide. Citation: Sano M, Uchida T, Igarashi M, Matsuoka T, Kimura M, Koike J, Fujisawa M, Mizukami H, Monma M, Teramura E, Yoshihara S, Sato H, Morimachi M, Ito A, Ueda T, Shiraishi K, Matsushima M, Suzuki T, Koga Y. Increase in the Lipopolysaccharide Activity and Accumulation of Gram-Negative Bacteria in the Stomach With Low Acidity. *Clin Transl Gastroenterol* 2020; 11: e00190. Copyright ©Wolters Kluwer Health, Inc. 2020. Published by Wolters Kluwer Health, Inc.

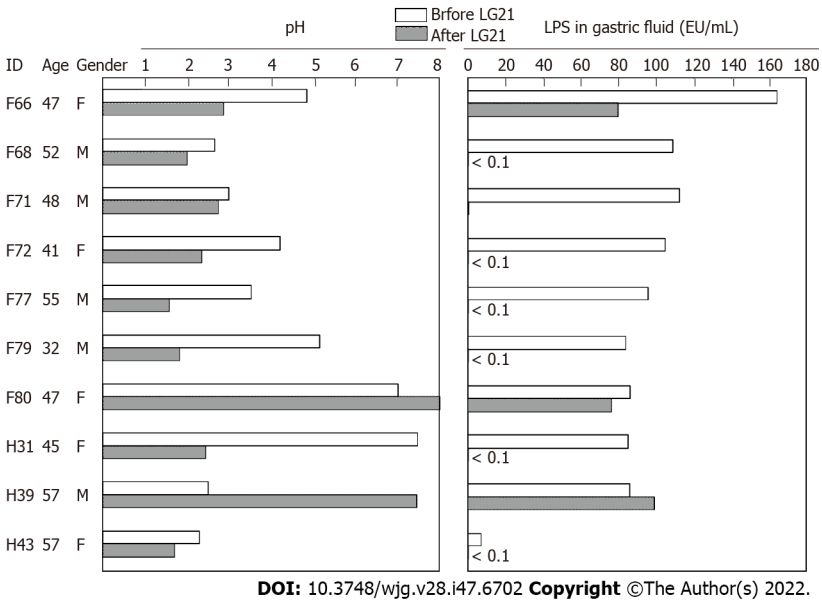

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Figure 4 Effect of LG21 administration on the pH and lipopolysaccharide activity in the gastric fluid. Ten subjects who had gastric fluid (GF) with low acidity and high lipopolysaccharide (LPS) activity consumed yogurt containing 10^9 CFU of LG21 every day for 3 mo. The pH value and LPS activity in the GF were measured before and after LG21 treatment. LPS: Lipopolysaccharide.

intestinal bacteria or an increase of bacterial species of oral cavity origin[47,48]. It seems unlikely that the deoxidization of dietary nitrates to nitrite by such dysbiotic bacteria could rapidly convert dietary amines into carcinogenic N-nitro compounds, because this conversion requires a sufficient amount of acid (which is not present in the stomach with mucosal atrophy)[49].

Sung *et al*[5] analyzed gastric microbes associated with gastric mucosal inflammation-which is considered to be the strongest risk factor for post-eradication gastric cancer-at one year after *H. pylori* eradication. They identified several of bacterial groups that were significantly associated with persistent inflammation. These bacteria included the genera *Acientobacter, Ralstonia, Actinobacillus* and *Erwinia,* which are all Gram-negative bacteria. Miyata *et al*[50] isolated several types of Gram-negative bacteria from the *H. pylori*-infected gastric mucosa, including *Fusobacterium, Haemophilus, Neisseria* and *Veillonella* species. Coculture of a gastric epithelial cell line with the lipopolysaccharide (LPS) specimens extracted from these bacterial groups stimulated a significant amount of IL-8 production. Sano *et al*[51] found high LPS activity in gastric fluid samples with weak acidity (pH > 4), whereas there was little or no activity in those with strong acidity (pH < 2). Spearman's test demonstrated a close correlation between

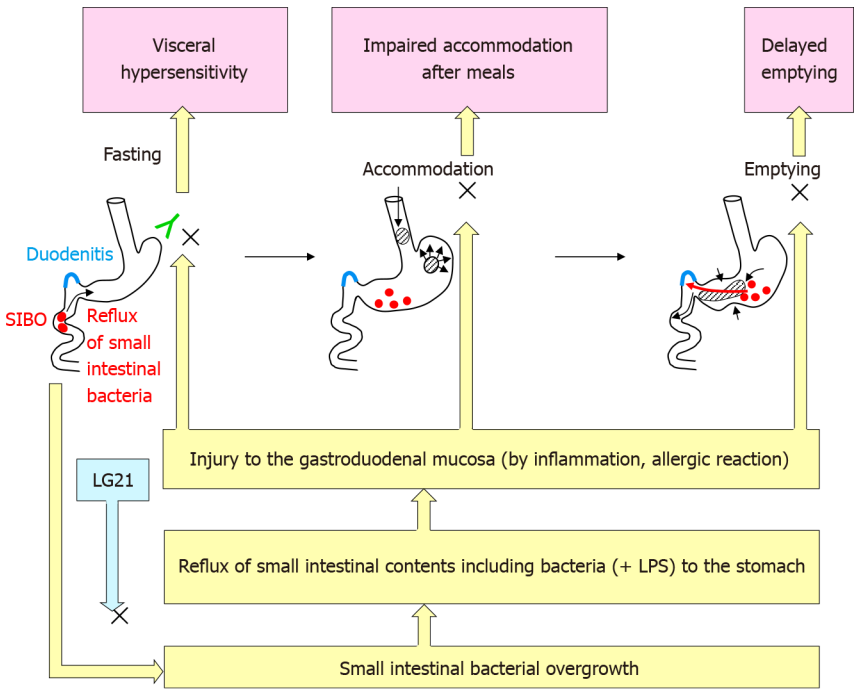

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Figure 5 Pathophysiology of functional dyspepsia and possible mechanisms of the effects of LG21 treatment. SIBO: Small intestinal bacterial overgrowth; LPS: Lipopolysaccharide.

pH and LPS activity in their 136 samples (r = 0.872) (Figure 3). These findings suggested that LPS from such non-*H. pylori* Gram-negative bacteria may perpetuate gastric inflammation and accelerate neoplastic progression in the hypochlorhydric stomach after *H. pylori* eradication.

To examine a possible preventive effect of probiotics on post-eradication gastric cancer, we administered a probiotic LG21 strain to subjects with successful eradication who still suffered from atrophic gastritis. In a fasting state in the morning, the pH value and LPS activity of their gastric fluids' samples were > 3.0 and > 10 EU/mL, respectively (Figure 4). Then, they received 10^9 CFU of LG21 in yogurt every day for 3 mo. In 8 of 10 subjects, the pH value considerably decreased after LG21 treatment. Lactic acid secreted by the probiotic LG21 strain is thought to restore acidity in the stomach with low acidity. Interestingly, the LPS activity of these subjects, in whom the gastric acidity partially recovered, almost disappeared or markedly decreased. The possible termination of LPS-induced inflammation by LG21 suggests a possible role of probiotics in preventing the development of gastric cancer after *H. pylori* eradication[52].

FD

FD is defined as the presence of symptoms that are thought to originate in the gastroduodenal region, in the absence of any organic, systemic or metabolic disease that is likely explain the symptoms. Because of the high prevalence and recurrent nature of symptoms, FD is a clinical problem of considerable magnitude for healthcare. According to the Rome IV criteria[53], there are two subtypes of FD: Postprandial distress syndrome (PDS) with postprandial fullness or early satiation, and epigastric pain syndrome (EPS) with epigastric pain or epigastric burning. The symptoms must be severe enough to affect daily activities, and must be present for > 3 mo with the onset of symptoms at least 6 mo before the diagnosis. While the exact pathophysiology of FD remains to be clarified, gastric motility disturbance (*e.g.*, impaired gastric accommodation and delayed gastric emptying) and visceral hypersensitivity have been suggested as critical underlying mechanisms (Figure 5)[54]. Recently, accumulating evidence supports that the duodenum is a target region underlying the pathophysiology of FD[55]. Impaired mucosal integrity and low-grade inflammation in the duodenum are thought to be associated with altered neuronal signaling and mucosal immune activation in this region. This eventually result in the uncontrolled motile and sensory mechanisms in FD. In addition, gastric acid, bile, food and microbiota are considered to induce and/or aggravate such underlying disorders in FD.

There is evidence to suggest that dysbiosis of intestinal microbiota is involved in the pathogenesis of irritable bowel syndrome (IBS) [a functional gastrointestinal disorder (FGID) originating in the intestine] [56]. However, the role of the gastroduodenal microbiota in the pathophysiology of FD (an FGID originating from the stomach and possibly the proximal small intestine) remains to be clarified. *H. pylori*

infection had been considered to be involved in the pathogenesis of FD-like symptoms that are often observed in these subjects. While FD-like symptoms in some *H. pylori*-infected patients are alleviated by antimicrobial eradication therapy, the improvement of the symptoms might not be mediated by the elimination of *H. pylori* but by the effect of antimicrobials on non-*H. pylori* bacteria in the stomach and proximal small intestine[57]. Indeed, Miwa *et al*[58] demonstrated that the curative treatment of *H. pylori* infection in eradication therapy was not significantly accompanied by the improvement of symptoms in a double-blind placebo-controlled clinical test. The involvement of an *H. pylori*-independent mechanism in the pathogenesis of FD is also suggested by a clinical study of probiotics. When the effect of an LG21 strain on FD-like symptoms was examined in *H. pylori*-infected patients, the severity of PDS after LG21 treatment was significantly lower than that was before treatment, while laboratory tests indicating the number and activity of *H. pylori* colonizing the stomach showed no significant difference between before and after the treatment[59]. These results suggested that bacteria other than *H. pylori,* which are resident in the GI tract, play an important role in the pathophysiology of FD. Tan *et al*[60] reported that the oral administration of the antimicrobial refaximin to patients with FD induced adequate relief of PDS. This implied the involvement of dysbiotic microbiota in the pathogenesis of FD. Nakae *et al*[61] compared the structure of the microbiota in GF between 44 FD patients and 44 healthy control subjects. A PERMANOVA test showed that the overall bacterial community structures of the two groups were significantly different (P = 0.001). In the bacterial composition analysis using those samples[62], the accumulation of bacteria that usually colonize the intestine, such as *Bacteroides*, *Bifidobacterium* and *Escherichia,* was often found in the GF of FD patients. As bile acids are also detected in these GF samples, the reflux of small intestinal contents, including bile and proximal small intestinal bacteria, to the stomach was suggested to induce such changes in the bacterial composition. Small intestinal bacterial overgrowth (SIBO) is broadly defined as an increase in the number of bacteria in the proximal small intestine together with various types of GI symptoms[63]. More than 60% of Japanese patients with FD have been reported to have overlapping IBS, in which SIBO is considered a critical etiological factor[64]. Thus, FD patients, whose GF contained large numbers of intestinal-type bacteria, might also suffer from SIBO. It is likely that the duodenal mucosa is injured by bile and/or the bacteria-especially Gram-negative bacteria such as *Escherichia*-in the content that is refluxed from the small intestine. Such mucosal damage would cause both deranged duodenal mucosal integrity and low-grade inflammation in the mucosa (Figure 5). Their studies also showed that 12-wk treatment of FD patients with an LG21 strain was effective for significantly improving symptoms, and shifted the composition of the GF microbiota to that observed in healthy subjects, whose GF microbiota no longer included any intestinal-type bacteria[61,62]. Therefore, the disappearance of such dysbiotic intestinal-type bacteria may be attributable to the resolution of SIBO after LG21 treatment.

Interestingly, there was also a significant inverse correlation between the differential abundance of *Prevotella* and the improvement of PDS symptoms in the FD patients treated with LG21[61]. That is; a greater increase in the relative abundance of *Prevotella* in GF after treatment was associated with a higher degree of symptom improvement. A significantly higher abundance of *Prevotella* on the duodenal mucosa was also observed in healthy control subjects in comparison to patients with FD[65]. Given that the genus *Prevotella* is sensitive to bile[66], the increase in the abundance after the treatment in FD patients may reflect a lower frequency of bile reflux, while lower abundance in FD patients may reflect a higher frequency of bile reflux.

To evaluate the clinical efficacy of treatment with a probiotic LG21 strain in FD patients, a randomized placebo-controlled clinical trial was performed using 116 individuals without *H. pylori* infection[67]. Participants were assigned to ingest yogurt containing 10^9 CFU of LG21 (LG21 group) or LG21-free yogurt (placebo group) every day. According to a questionnaire on the severity of FD symptoms, a trend toward a positive overall ameliorative effect on FD symptoms was observed in the LG21 treatment group (P = 0.07). Moreover, after treatment, the elimination rate for 4 major FD symptoms (postprandial fullness, early satiety, epigastric pain and epigastric burning) was 17.3% in the placebo group and 35.3% in the LG21 group, respectively (P = 0.048).

Although the beneficial effect of LG21 on FD was demonstrated by the clinical trial, the underlying mechanism remains to be clarified. One possible mechanism is protection of the duodenal mucosa from injurious intestinal bacteria and bile by through the resolution of SIBO and/or frequent duodeno-gastric reflux, as mentioned above (Figure 5). Considering that acid-suppressive therapy is so effective and has thus been recommended as the first-line treatment for FD[68], another mechanism underlying the effects of LG21 treatment may be a reduction of gastric acid production. Nakae *et al*[61], reported that the mean pH values (IQR) of GF of FD patients (n = 44) before and after the treatment were 1.58 (1.43-1.85) and 1.84 (1.56-3.81), respectively. Although this difference was not so great, it was statistically significant (P = 0.012). Given that hypersensitivity of the gastroduodenal mucosa is a critical pathophysiology underlying FD, it is reasonable that even a small reduction of gastric acidity by LG21 treatment can attenuate the gastric sensory and motor disturbances, which would then lead to an improvement of PDS and EPS symptoms. Similarly, in addition to PPI treatment, H-2 receptor antagonist (H2RA) treatment is effective for FD, although the efficiency of acid suppressive by H2RA is considerably lower in comparison to PPIs[55]. The moderate decrease in the secretion of gastric acid observed with LG21 treatment may be attributable to a reduction in the expression of gastrin (an acid secretion stimulating hormone) as the oral administration of LG21 has been reported to reduce the gastrin exression in the

murine system[69]. However, LG21 treatment did not reduce the serum gastrin concentration at all in long-term PPI users, who showed very high gastrin levels (> 200 pg/mL)[70]. This means that the suppressive effect of LG21 is no longer exerted in subjects with high gastrin levels such as PPI users and possibly patients with corpus-dominant atrophic gastritis, in whom the serum gastrin concentration is abnormally high due to the secondary response to very low intragastric acidity. Emerging data increasingly point toward the role of gastroduodenal microbiota in the pathophysiology of FD. Accordingly, the application of probiotics in the treatment of this regions is expected to be successful.

CONCLUSION

Probiotics for the stomach have been demonstrated to suppress *H. pylori* in the stomach, and thus improve eradication rate in patients who receive antimicrobial treatment. If probiotics strains are sufficiently resistant to the gastric acidity and able to achieve close proximity to the gastric mucosa, they are also expected to prevent the development of gastric cancer, even after *H. pylori* eradication, through the correction of the dysbiotic gastric microbiota. If a deranged gastric bacterial population is involved in the pathophysiology of functional dyspepsia, the use of such probiotics may be useful for the treatment of this functional gastroduodenal disorder.

FOOTNOTES

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MINIREVIEWS

Liver injury in COVID-19: A minireview

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Abstract

Coronavirus disease 2019 (COVID-19), caused by infection with the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), has escalated into a global tragedy afflicting human health, life, and social governance. Through the increasing depth of research and a better understanding of this disease, it has been ascertained that, in addition to the lungs, SARS-CoV-2 can also induce injuries to other organs including the liver. Liver injury is a common clinical manifestation of COVID-19, particularly in severe cases, and is often associated with a poorer prognosis and higher severity of COVID-19. This review focuses on the general existing information on liver injury caused by COVID-19, including risk factors and subpopulations of liver injury in COVID-19, the association between pre-existing liver diseases and the severity of COVID-19, and the potential mechanisms by which SARS-CoV-2 affects the liver. This review may provide some useful information for the development of therapeutic and preventive strategies for COVID-19-associated liver injury.

Key Words: Liver; SARS-CoV-2; Angiotensin-converting enzyme 2; Transmembrane serine protease 2; Chronic liver disease

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Core Tip: The global pandemic of coronavirus disease 2019 (COVID-19) has imposed a great threat to human health and become a medical and social challenge. Although severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) mainly affects the respiratory tract, it also frequently damages the liver especially in severe and critical cases. Direct hepatotoxicity of SARS-CoV-2, or indirect hepatic injury caused by immune overactivation and systemic inflammation, drug-induced injury, ischemia/reperfusion and hypoxia/reoxygenation injuries, and worsening of preexisting liver diseases, are potential contributing factors to liver damage in COVID-19.

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INTRODUCTION

In December 2019, an outbreak of the novel coronavirus disease 2019 (COVID-19) was reported in Wuhan, Hubei Province, China, induced by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). COVID-19 has evolved into a global health challenge[1], posing an enormous threat to human health and economic development. Severe COVID-19 patients may present symptoms of acute respiratory distress syndrome (ARDS), requiring admission to the intensive care unit (ICU) and oxygen ventilation therapy. The shortest time from admission to ARDS is approximately 2 d. At this stage, COVID-19 mortality is extremely high[2]. Global SARS-CoV-2 infection is now widespread, with 601189435 confirmed COVID-19 cases, including 6475346 deaths reported until September 3, 2022 by the WHO[3]. Most COVID-19 patients exhibit mild symptoms (fever, cough, shortness of breath, fatigue, vomiting, diarrhea, anosmia, and headache), whereas critical cases may develop into severe illness and even death due to severe lung injury and respiratory failure, liver injury, cardiac injury, septic shock, and even multi-organ failure[4].

Although COVID-19 mainly affects the respiratory tract, researchers have focused on the impacts of SARS-CoV-2 on other organs[5]. Liver injury is common in COVID-19 and is associated with poor prognoses. Liver test abnormalities, including higher levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), are frequently observed in severe and critical COVID-19 cases compared to mild and moderate cases[6]. The most common manifestation of liver injury in COVID-19 is acute hepatitis with elevated AST, ALT, and total bilirubin levels[7]. Moderate microvascular steatosis and slight lobular activity are commonly observed in the liver biopsies[8]. Possible mechanisms include direct SARS-CoV-2 infection in hepatocytes or bile duct epithelial cells, excessive inflammation, drug-induced liver injury (DILI), ischemia/reperfusion syndrome, and liver injury associated with preexisting liver disease. Angiotensin-converting enzyme 2 (ACE2) is known to be the most important receptor for SARS-CoV-2 in contact with the host cell membrane, while transmembrane serine protease 2 (TMPRSS2) also plays an essential role in mediating viral entry. TMPRSS2 can prime the spike protein (S protein), and has been considered as a target for designing TMPRSS2 inhibitors to block virus entry as a new therapeutic approach[9]. When cells are infected by SARS-CoV-2, the S protein of the viral particles must be cleaved by TMPRSS2 and then binds to ACE2, so that viral particles can fuse with the plasma membrane and enter host cells[10]. Lower ACE2 expression has been detected in hepatocytes, while biliary epithelial cells express abundant ACE2 and thus can serve as a binding site for SARS-CoV-2. It has also been shown that most viruses that infect the respiratory tract can damage hepatocytes by affecting the $CD8^+$ mediated immune response[11]. Common risk factors, such as older age, male sex, and a range of potential comorbidities, including hypertension, obesity, diabetes, and underlying liver diseases, can also lead to varying degrees of liver damage, critical illness, and even death. However, the exact connection between comorbidities and liver injury caused by SARS-CoV-2 remains unclear. This review summarizes the pathophysiology, possible mechanisms, clinical manifestations, risk factors, and special populations of liver injury in COVID-19 patients.

PATHOPHYSIOLOGY OF LIVER INJURY IN COVID-19

The mechanism of liver injury in COVID-19 remains largely unknown. SARS-CoV-2 may induce direct hepatotoxicity after entering into the liver *via* bile duct cells which express high levels of the ACE2 receptor. SARS-CoV-2 may also indirectly injure the liver *via* immune overactivation, systemic inflammation, drug toxicity, and hepatic hypoxia/reoxygenation or ischemia/reperfusion due to respiratory failure and endothelial damage (Figure 1). The liver is the main organ for detoxification and metabolism, and liver injury can reflect the severity and clinical course of COVID-19. Therefore, it is

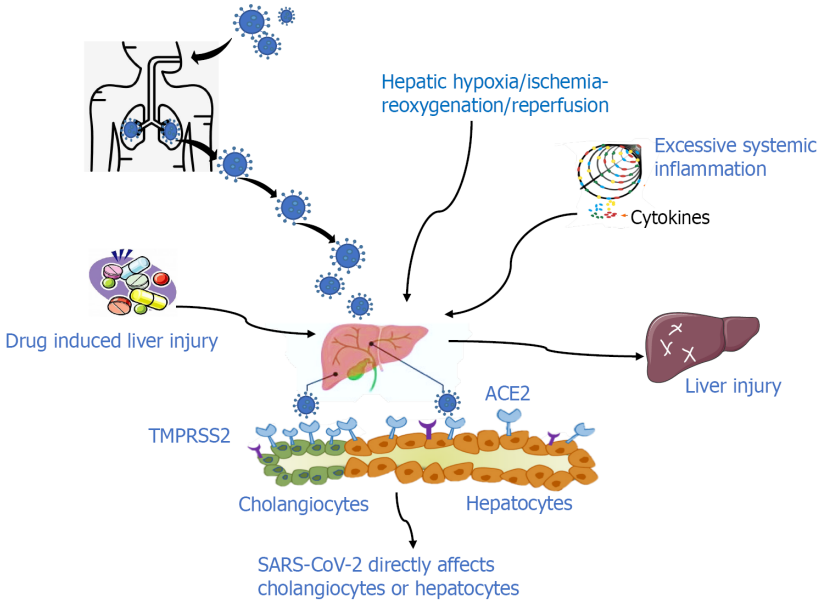

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Figure 1 Mechanisms of liver injury caused by SARS-CoV-2. The mechanisms include direct hepatoxicity (severe acute respiratory syndrome coronavirus 2 affects cholangiocytes or hepatocytes) or indirect hepatic injury (drug-induced liver injury, excessive systemic inflammation and cytokine storm, and deterioration of pre-existing liver disease). SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; ACE2: Angiotensin-converting enzyme 2; TMPRSS2: Transmembrane serine protease 2.

crucial to understand the mechanisms underlying liver injury in COVID-19 to develop effective treatments.

Direct effect of SARS-CoV-2 on the liver

Previous research has established that the ACE2 receptor can specifically bind to the S protein, which has a receptor binding domain (RBD) to mediate SARS-CoV-2 entry into host cells. Although the binding pattern of the SARS-CoV-2 RBD-ACE2 complex is greatly analogous to that of the SARS-CoV RBD-ACE2 complex, ACE2 has a higher affinity for the RBD of SARS-CoV-2[12]. This may be one possible reason why the SARS-CoV-2 is more dangerous than the other emerging SARS-CoVs. However, the level of ACE2 in the liver tissue is much lower than that in the bile duct based on previous data analysis, which suggests that SARS-CoV-2 may bind to ACE2-positive bile duct cells, but not hepatocytes[13]. Another possibility is that ACE2 can sense viral entry and upregulate its expression in hepatocytes[14]. Current evidence indicates that bile duct cells are actively involved in immune defense, inflammatory response, and liver regeneration, which may be a possible explanation for virus-induced liver injury once these cells are damaged. Cholangiocytes can co-express ACE2 and TMPRSS2, and are susceptible to viral infections[13]. Elevated levels of gamma-glutamyl transpeptidase (GGT) and alkaline phosphatase (ALP) are caused by bile duct cell injury[15]. A previous study reported the histological, ultrastructural, and immunohistochemical staining of liver biopsies performed on two patients who died of COVID-19. The researchers discovered a mass of viral particles of SARS-CoV-2 in the hepatocyte cytoplasm, and most of the viral particles had an intact coronoid envelope, indicating that SARS-CoV-2 can not only enter, but also replicate in the hepatocytes[14]. Another report also presented detailed liver histological results from two patients with acute COVID-19, and found extensive mitosis especially in the cholangiocytes, in addition to mixed inflammatory infiltrates in the portal region, endodermatitis, and severe bile duct injury[16]. Another study of three patient cohorts provided evidence of SARS-CoV-2 liver tropism. In autopsy reports from the third cohort of patients, viral RNA was detected in 69% of autopsy liver specimens and SARS-CoV-2 carrying infectiousness was detected in post-mortem liver tissues[17]. Regardless of whether SARS-CoV-2 directly affects cholangiocytes or hepatocytes, all the above studies support that the liver injury caused by SARS-CoV-2 is a direct cytopathic injury[16] (Figure 2A). Furthermore, obvious mitochondrial swelling, endoplasmic reticulum dilatation, and impaired cell membranes were also observed in two COVID-19 cases, suggesting cytopathic damage. However, pathological changes including moderate microvascular steatosis and slight lobular and portal inflammatory infiltration are non-specific in viral infection and can be caused by DILI or chronic liver disease (CLD) such as non-alcoholic fatty liver disease (NAFLD) [18]. In addition, no viral inclusion bodies were observed in the liver tissue of COVID-19 patients. In summary, it is still unclear whether SARS-CoV-2 directly causes cytopathic changes in liver cells.

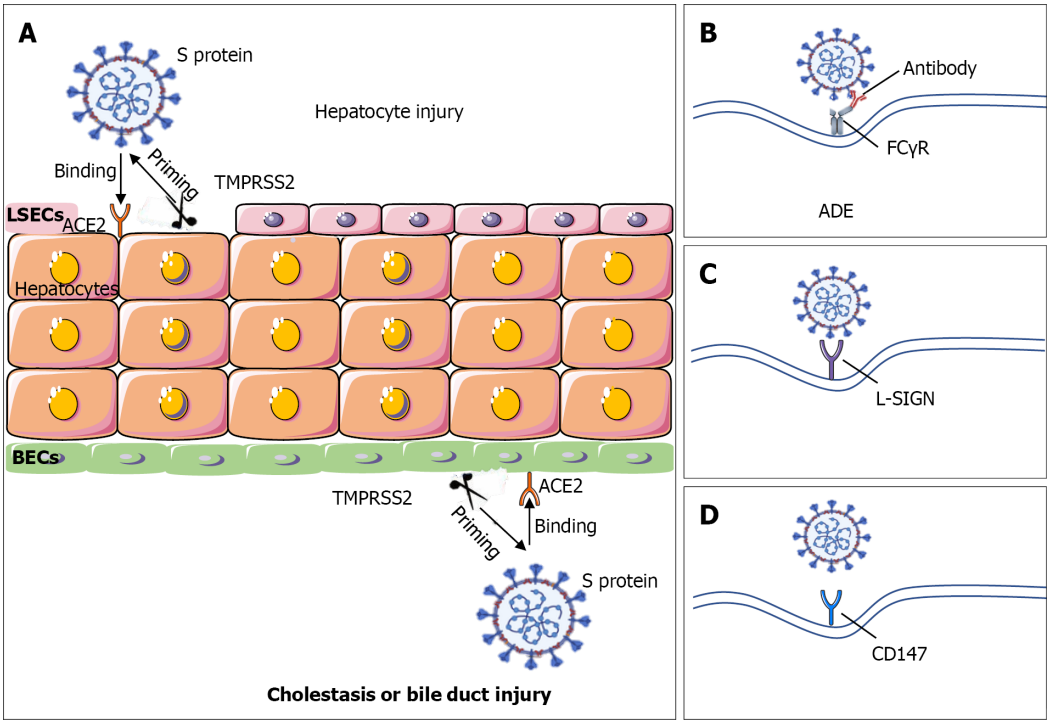

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Figure 2 Possible pathways of SARS-CoV-2 entering into the liver. A: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infects host cells through angiotensin-converting enzyme 2 (ACE2) and transmembrane serine protease 2 (TMPRSS2) receptors by its S protein. Direct SARS-CoV-2 infection targeted to hepatocytes or billiary epithelial cells results in hepatocyte injury or bile duct injury; B and C: CD147 (B) and L-SIGN (C) may be alternative receptors for SARS-CoV-2 entry into the liver; D: Antibody dependent enhancement is a pathway which can enhance interaction of virus-based antibody and the CR and/or FC receptor complements further making virus easily entry and infection. ACE2: Angiotensin-converting enzyme 2; TMPRSS2: Transmembrane serine protease 2; LSECs: Liver sinusoidal endothelial cells; BECs: Biliary epithelial cells.

Systemic inflammatory response and cytokine storm in COVID-19

Severe COVID-19 is characterized by a systemic inflammatory response that may cause a cytokine storm leading to multi-organ failure. Current evidence has shown that serum inflammatory cytokine levels are positively correlated with the indicators of liver dysfunction in COVID-19 patients[19]. This suggests that systemic inflammatory response and cytokine storm are also involved in liver injury, with a possible underlying mechanism between them. A previous study has shown that the activation and dysregulation of $CD8^+$ T cells in severe patients may be an important factor for the pathogenesis of SARS-CoV-2 infection, as $CD8^+$ T cells in critically ill patients express high levels of cytotoxic molecules [20]. A cohort study of 133 COVID-19 patients with liver damage reported high levels of inflammatory cytokines [TNF-α, interleukin (IL)-2, IL-6, and IL-10] and low levels of T lymphocyte subsets ($CD3^+$, $CD4^+$, and $CD8^+$ T cells). IL-2, IL-6, IL-10, $CD4^+$, and $CD8^+$ T cells can be regarded as possible independent predictors of hepatic injury in COVID-19 patients[21]. This is consistent with an alternative study, which also revealed that the elevated levels of IL-2, IL-6, and IL-10 in the serum of COVID-19 patients were associated with the progression of severe disease[22]. Notably, IL-6 is particularly important for liver injury, because IL-6-mediated procoagulant endotheliopathy with increased hepatic von Willebrand factor (vWF) expression and platelet accumulation are linked to liver injury and liver inflammation (elevated ALT and neutrophil infiltration). In addition, IL-6 plays a potential role in hepatic endothelial dysfunction and inflammation because its level is correlated with vWF level[23]. IL-6 is associated with elevated liver enzymes, but the relationship is not necessarily causal, as IL-6 can be used to accurately detect inflammatory responses to liver injury[24]. IL-6 is activated primarily by the pathway of JAK (Janus kinase)/STAT (signal transducer and activator of transcription). Baricitinib, an inhibitor of the JAK/STAT pathway, can improve the clinical outcomes of COVID-19[25]. JAK inhibition can affect the viral entry and inflammation of COVID-19[26]. Previous studies have established an association between increased levels of endotoxin, ILs, and TNF-α in COVID-19 patients with liver function damage compared with those with normal liver function[27]. COVID-19 patients with elevated ALT levels also have increased IL-6, ferritin, lactate dehydrogenase (LDH), and C-reactive protein (CRP) [14]. Furthermore, significantly increased levels of IL-2, IL-7, IL-10, granulocyte colony-stimulating factor (G-CSF), and IP-10 (interferon-inducible protein-10) have been observed in severe patients compared with mild or moderate patients[22]. In conclusion, patients with severe COVID-19 may exhibit intense inflammation and cytokine storm syndrome leading to liver injury.

DILI in COVID-19

An additional opinion is that liver injury in COVID-19 patients is related to hepatotoxicity. DILI cannot be overlooked, as it may contribute to abnormal liver function, such as elevated ALT and ALP levels, and subsequently affect drug metabolism and excretion[28]. In addition, histopathologic findings from liver biopsies of COVID-19 patients, such as microvascular steatosis and liver inflammation, may also be associated with DILI[18]. Recent evidence suggests that DILI may occur secondarily to the drugs commonly used for COVID-19 treatment, such as paracetamol, antiviral therapies, low molecular weight heparin, anti-IL-6 receptor agents, and antibiotic treatments[29]. This is consistent with another study, in which corticosteroids and immune modulators were also mentioned[18]. Notably, NAFLD patients are more likely to develop DILI, because NAFLD can increase the sensitivity of the liver to hepatotoxicants, such as acetaminophen[30]. Antibiotics and nonsteroidal anti-inflammatory drugs are considered one of the most common causes of DILI[31]. One previous study showed that, when receiving glucocorticoid therapy, more patients had a liver injury (58.1%) than those without (39%)[32], and, tocilizumab (TCZ)-induced liver injury was reported in one COVID-19 patient. TCZ, also known as an IL-6 inhibitor, is recommended for the treatment of COVID-19 due to its vital role in inducing cytokines, and increased IL-6 can predict the fatal outcome of COVID-19. In this case, when TCZ was administrated, serum aminotransferase levels increased by nearly 40-fold on the first day, and after 10 d of DILI formation, aminotransferase levels returned to normal. Therefore, it is no doubt that TCZ has positive effects on other clinical and laboratory parameters of cytokine storm, such as CRP, IL-6, fibrinogen, and D-dimer, resulting in transaminase levels close to the normal range[33]. In brief, it is necessary to emphasize the importance of drug-related liver damage in patients with COVID-19, especially in those with underlying liver diseases.

Hypoxia/reoxygenation and ischemia/reperfusion-induced liver damage

COVID-19 is primarily characterized by respiratory failure, thus, severe cases of hypoxic hepatitis are commonly seen, and 10% of the cases suffer from a hypoxic liver injury in the ICU[34]. Hypoxic hepatitis, also known as ischemic hepatitis or shock liver[35], is accompanied by a rapid elevation of aminotransferases in cases of respiratory failure, shock, or heart failure. One possible reason is that the complex vascularization of the liver makes it susceptible to changes in circulation, resulting in decreased liver perfusion[31]. It has been shown that SARS-CoV-2 also causes liver damage by producing diffuse endodermatitis. Viral inclusion structures can also be observed in endothelial cells. Liver ischemia/reperfusion, including ischemia-induced cell injury and reperfusion-induced inflammatory responses resulting from the activation of neutrophils, Kupffer cells, and platelets, can induce the generation of reactive oxygen species (ROS) and calcium overload. Endothelial cells are involved in liver ischemia/reperfusion damage, which can promote oxidative stress through ROS and derivatives of nitric oxide[18]. ROS and lipid peroxidation products can mediate the production of redox-sensitive transcription factors, which in turn induce the release of abundant pro-inflammatory factors, resulting in hepatic injury[31]. Furthermore, hepatic sinusoidal endothelial cell damage has been reported to further aggravate hepatic ischemia and hypoxia by disturbing microcirculation[36]. In addition, a high level of positive end-expiratory pressure may be a possible contributor to liver injury in COVID-19 patients, because it can increase right atrial pressure and obstruct venous return, leading to hepatic stasis[37]. These findings indicate that hepatic hypoxia/reoxygenation and ischemia/reperfusion may be potential etiologies of COVID-19-related liver injury.

The ACE2-independent pathway of liver injury

In addition to receptor-mediated viral entry, antibody dependent enhancement (ADE) may also partially associated with hepatic injury in COVID-19 patients. ADE is a pathway that can enhance the interaction of virus-based antibodies, and the CR and/or FC receptor complements allow the virus to easily come into contact with macrophages, granulocytes, and monocytes. This results in the virus multiplying and increasing production, causing the infection to worsen. It has been identified that ADE can be activated by SARS-CoV antibodies, thus, SARS-CoV can trigger ADE activity in immune cells, even though the immune cells do not express ACE receptors (Figure 2B). However, there is a lack of discussion concerning the ADE pathway of liver injury, thus the ACE2-independent mechanisms of liver damage in COVID-19 remain unclear and warrant further study[27].

There is alternative hypothesis that liver cells express two receptors, CD147 and L-SIGN, which have an affinity for the S protein of SARS-CoV-2, and thus may mediate the infection of liver cells by SARS-CoV-2. The presence of CD147 on the surface of host cells may be a new way for SARS-CoV-2 invasion [38]. It has been experimentally verified that CD147 directly interacts with SARS-CoV-2 and that blocking CD147 can prevent SARS-CoV-2 replication, whereas massive expression of CD147 can promote SARS-CoV-2 replication[39]. These findings suggest that CD147 mediates the entry of SARS-CoV-2 into liver cells. However, this requires further exploration (Figure 2C). Additionally, L-SIGN serves as a liver-specific membrane receptor associated with viral capture. Autopsy studies have shown that L-SIGN receptors were present in SARS-CoV-2-infected sinusoidal cells[40] (Figure 2D). Therefore, L-SIGN may provide an alternative way for SARS-CoV-2 to invade the liver tissue.

Clinical manifestation of liver injury in COVID-19

Liver injury is common in severe COVID-19 cases, as shown by significant circulatory elevations in hepatocyte enzymes including ALT and AST, and a slight increase in cholangiocyte-related enzymes such as GGT and ALP. Mao *et al*[41] analyzed 35 studies involving 6686 COVID-19 patients, among which one in five patients developed liver function abnormalities, especially in severe cases. Therefore, paying close attention to liver function in COVID-19 patients before and during admission is necessary to control the severity of COVID-19. The main findings of the liver biopsy were moderate microvascular steatosis and mild lobular and portal activity. Other specific clinical manifestations of liver injury warrant further investigation.

Abnormal liver function tests

Liver test abnormalities are frequent in COVID-19 patients and often progress to severe illness. Liver function tests include hepatocyte injury markers ALT and AST, cholestasis or bile duct injury markers GGT and ALP, and measures of synthetic capacity [prothrombin time (PT) and albumin (ALB)]. One comprehensive study has described the results of liver tests from 417 patients with COVID-19 and found elevated levels of ALT and GGT (24%), AST and total bilirubin (TBIL, 12% and 15%), and no increase in ALP[42]. Such elevated GGT and normal ALP cannot be attributed to bile duct type but are possibly drug-induced liver injuries. GGT elevation is also considered to be a marker of substitution of excessive oxidative stress and increased inflammation due to acute inflammatory stress. However, by analyzing a small subset of COVID-19 patients with possible persistent abnormalities over 60 d, one study found that the predominant abnormality was a cholestatic pattern, as indicated by elevation of the ALP/GGT ratio[43]. Although GGT is not a specific marker of liver injury, liver damage may result from direct bile duct cell injury and cholestasis induced by SARS-CoV-2[43]. Many studies in China revealed that COVID-19 patients who used lopinavir/ritonavir during hospitalization had liver injuries that influenced liver tests[44]. A cohort study including 1040 COVID-19 patients (mean age 38 years old, 54% men) revealed that drugs used for treatment, such as lopinavir-ritonavir, interferon beta, and/or corticosteroids, are correlated with an elevation in the ALT/AST ratio, which acts as an independent contributor to worse clinical outcomes[45]. Using a random effect method and an inverse variance approach, a meta-analysis was conducted using 128 studies, identifying that the most common abnormalities were hypoalbuminemia [61.27% (48.24-72.87)], increased levels of GGT [27.94% (18.22-40.27)], ALT [23.28% (19.92-27.01)] and AST [23.41% (18.84-28.70)][46]. Notably, hypoalbuminemia is an independent risk predictor of liver dysfunction and mortality upon admission[47]. One previous study analyzed 2623 confirmed COVID-19 cases at different risk levels, including non-critically ill, critically ill, and deceased groups[48]. This study revealed that hypoalbuminemia may be due to hepatotoxicity of the cytokine storm. In addition, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) levels were significantly lower in the critically ill and dead groups than in the non-critically ill group. Furthermore, patients with abnormal liver function had higher inflammatory indices, such as a high level of CRP which is associated with disease severity. Patients with an increased level of ALP have significantly elevated CRP levels, and CRP levels are correlated with ALT levels[49]. Notably, D-dimer, LDH, creatine kinase, and troponin levels are also higher in patients with abnormal liver function tests than in those with normal liver function[50]. A retrospective study analyzed 216 patients diagnosed with COVID-19 and concluded that elevated LDH is an independent contributing factor to ICU admission and may require mechanical ventilation[51]. However, the increase in serum LDH does not specifically reflect cellular damage in the liver, because LDH is present in many organs.

In summary, abnormal liver function tests are frequent in COVID-19 patients, which are secondary to other injuries, mostly ischemia or drug-induced liver damage. Patients with abnormal liver tests have extended hospital stay and are at higher risk of developing severe disease; however, this does not directly leading to death[52].

Hepatic histopathological findings in COVID-19

Hepatic histopathological findings in patients who died from COVID-19 revealed moderate microvascular steatosis in addition to mild lobular and portal activity. This is consistent with other studies suggesting that the changes are non-specific, and liver injury may be due to SARS-Cov-2 or drugs, but not caused by cholestasis[8,53,54]. Some studies have found sinusoidal thrombosis, which is less common and is mostly observed in severe COVID-19, because COVID-19 is a prothrombotic disease [55,56]. A case study of the liver autopsy of an elderly patient with COVID-19 found no obvious inflammation in the portal area, but observed a few hepatocytes with slight vesicular steatosis and watery degeneration, possibly associated with ischemia and hypoxia[42]. Inflammatory cells, including neutrophils, plasma cells, and Kupffer cells, were observed in the hepatic sinuses at a higher magnification. In addition, numerous scattered or apoptotic hepatocytes characterized by nuclear condensation or formation of apoptotic bodies were also observed in liver biopsy tissues, but no viral inclusions were observed[14], similar to the results of Wang *et al*[32]. Based on these studies, direct effect of SARS-CoV-2 on hepatocytes seems less convincing. Notably, a report on the histopathological findings of 40 patients who died from COVID-19 revealed that mild lobular necroinflammation was present in 20 (50%) cases, with few infiltrations of lymphocytes and histiocytes[55]. Hepatocyte

degeneration with focal necrosis and low infiltrations of leukocytes in the lobular and portal areas have also been observed in an alternative liver biopsy study of deceased COVID-19 patients[57]. Liver biopsies from three patients and necropsies from three others revealed striking iron accumulation in hepatocytes, and an abundance of ferritin particles in damaged mitochondria[58], suggesting that hepatic iron overload could have a potential relationship with liver injury. Post-mortem wedge liver tissues from 48 deceased COVID-19 patients suggest that different degrees of liver injuries are present, including liver blood involvement and acute (thrombosis and lumen expansion) and chronic (fibrous thickening of vessel wall and portal vein fibrosis) liver injuries[59]. In conclusion, the most common seen liver histopathological findings were hepatic steatosis, portal inflammation, and varying degrees of liver injury. However, related investigations into the mechanisms and etiologies of hepatic histopathological changes are still lacking.

Other special clinical manifestations

In addition to the elevated liver enzymes and histopathologic changes described above, other specific manifestations of liver damage in COVID-19 patients have also been reported, including a darkened face and pigmentation. Pigmentation may be a result of abnormal liver metabolism *via* the following mechanisms. First, liver dysfunction can increase estrogen levels, thereby damaging the inhibitory effect of thiamine on tyrosinase, so that more tyrosine is transformed into melanin. Second, abnormal liver function may result in hypofunction of adrenocortical hormones, and melanin secretion is increased due to the elevation of the melanocyte-stimulating hormone. In addition, more iron may enter into the blood vessels due to liver damage, increasing the ion level in the blood circulation to the facial skin and resulting in a darkened face[27].

COVID-19-INDUCED LIVER INJURY IN SPECIAL POPULATIONS

Risk factors of severe COVID-19 and liver injury

A growing body of studies suggests that male sex, older age, and potential comorbidities, such as hypertension, obesity, diabetes, cardiovascular diseases, and respiratory diseases, and particularly obesity and type 2 diabetes, are more likely to be associated with critical COVID-19 after infection by SARS-CoV-2[60]. One study involving 174 consecutive COVID-19 patients shows that 24 of them had no other comorbidities but diabetes. By comparing the diabetic group with the non-diabetic group, it was found that COVID-19 patients with diabetes more easily experienced an overactivated inflammatory response and a hypercoagulable state, resulting in a worse COVID-19. The mechanism could be that COVID-19 patients with diabetes are easy to develop inflammatory storm that rapidly worsens COVID-19 due to high blood levels of inflammatory factors, such as IL-6, CRP, serum ferritin, coagulation index, and D-dimer[61]. These proinflammatory cytokines were strongly correlated with liver injury, so it is worth paying attention to whether patients with a history of diabetes are more susceptible to liver injury. Age is by far the most associated risk factor of COVID-19, with the risk increasing gradually after 65 years[62]. This may be due to T cell and B cell functions which show age-dependent defects, as well as inadequate control of viral replication due to overproduction of type 2 cytokines and prolonged inflammatory responses[63]. Compared to infected adults, infected children usually present with mild clinical symptoms, and are mostly asymptomatic[64]. This may be because of their immature immune systems. Past reports have confirmed that children with abnormal liver function tests such as increased ALT usually have a preexisting condition such as an immunocompromised state (including malignancy) or CLD[65]. Patients with poor underlying liver conditions, such as cirrhosis, hyperplasia, non-alcoholic steatohepatitis, and mild steatosis, could have high expression of ACE2 than those without liver diseases[66]. Preexisting liver disease and age ≥ 60 years are risk factors for progressing to severe COVID-19[67]. Besides, male sex is linked to the severity of COVID-19 and has been identified as an important etiology of liver injury, possibly due to the lack of the protective effect of estrogen on the liver [68], or the enhancement of male innate immunity and the activation of T cells[62].

Notably, patients with obesity represent a dysregulated hepatic innate immunity, adaptive immune response, and pro-inflammatory state, thus aggravating the cytokine storm caused by SARS-CoV-2 infection, resulting in worse prognoses of COVID-19[60]. To evaluate the relationship between obesity and COVID-19, a meta-analysis was conducted using 50 studies in total with a total of 18260378 eligible subjects. It was found that obesity increased the chance of contracting SARS-CoV-2 and developing critical COVID-19. In addition, patients with a body mass index (BMI) ≥ 30 were 1.39 times more susceptible to SARS-CoV-2 infection compared with those with normal body weight, and the hospitalization rate of COVID-19 became higher with increasing BMI[69]. However, fatty liver is not a predisposing factor for hepatic injury after SARS-CoV-2 infection compared with the susceptibility rate of the general population[70].

In addition to the above risk factors, chronic heart disease, excessive inflammatory response, extended prothrombin time (PT), elevated liver enzymes, and high levels of bilirubin may also be related to severe COVID-19 and mortality[71]. In conclusion, the sex of the patient (males), older age, and comorbidities were independent risk factors for the rapid aggravation of COVID-19. Patients older

than 60 years with a history of diabetes have an increased risk of mortality[72]. However, the exact association between liver injury and risk factors remains unclear.

Preexisting liver diseases and severity of COVID-19

CLD is deemed to be a major burden of disease and a threat to human worldwide. CLD mainly includes cirrhosis, alcoholic liver disease, non-alcoholic fatty liver disease, and chronic hepatitis B, which affects nearly 300 million people in China. Because of this high burden, it is necessary to carefully assess the relationship between underlying liver disease and liver injury in patients with COVID-19[73]. However, the extent to which CLD affects the liver function and the disease ending in COVID-19 is controversial. On the one hand, patients with CLD are thought to be more susceptible to viral infection and developing liver injury due to an altered immune status. On the other hand, previous studies reveal that CLD is rarely related to the progression of liver injury or critical/fatal outcomes of COVID-19 (Table 1)[74]. Therefore, the connection between the underlying liver disease and COVID-19 requires further investigation.

Cirrhosis and COVID-19

Cirrhotic patients have complex immune disorders and are more susceptible to infection, and the leading causes of mortality are not only respiratory complications but also deterioration of liver function, leading to end-stage liver disease. Patients with cirrhosis are not only at risk of related immune dysfunction, but also are more likely to have comorbidities that predispose them to severe COVID-19, such as diabetes, chronic kidney disease, and heart disease[75]. However, there is a debate about the risk of cirrhosis on the clinical outcomes of COVID-19. A multicenter retrospective study revealed that cirrhotic patients infected with COVID-19 have a high 30-d mortality rate[76]. In this study, elevated transaminases may have had an adverse influence on the process of cirrhosis, and ALT levels in cirrhotic patients were affected by SARS-CoV-2. Additionally, nearly half of the patients who previously had normal transaminases developed to an acute liver injury. Indeed, patients with impaired liver function have a higher 30-d mortality rate and more studies suggested that cirrhotic patients are more susceptible to infection resulting from immune dysfunction. SARS-CoV-2 damages the lymphocytes, especially T cells, resulting in impaired immune function and increased sensitivity to the viruses. Moreover, an elevated neutrophil-to-lymphocyte ratio (NLR) increases the risk of bacterial infection, which is related to stress and sepsis, particularly in decompensated liver cirrhosis leading to a worse outcome[67]. In a study of 386 patients with cirrhosis, 32% had a growing risk of mortality after SARS-CoV-2 infection. Patients with CLD without cirrhosis just have a similar risk of mortality as patients without liver disease[77]. In addition, cirrhotic patients with SARS-CoV-2 infection appear to have a high risk of developing acute hepatic decompensation (46%), as well as a 2-fold increased rate of mortality. These studies suggest that cirrhosis is closely linked to the disease progression from COVID-19.

Contrary opinions argue that CLD and cirrhosis have little correlation with the critical and mortality rates of COVID-19. One study found that there was no increase in liver damage after SARS-CoV-2 infection in CLD patients[18]. However, the results may be relatively low persuasive, because only three patients with cirrhosis were studied.

Hepatitis B and COVID-19

Recent evidence suggests that liver injury in patients co-infected with SARS-CoV-2 and hepatitis B virus (HBV) leads to increased disease severity and worse clinical outcomes[78]. In addition, liver test abnormalities often appear in patients co-infected with SARS-CoV-2 and HBV, with elevated levels of ALT, AST, ALP, and TBIL.

In addition, the largest multicenter retrospective study revealed several novel risk factors such as high levels of LDH, D-dimer, and reduced ALB or ALB/globulin (GLO), which increase the risk of COVID-19 severity and mortality in patients with chronic hepatitis B (CHB)[79]. An alternative study showed similar results: High levels of LDH, D-dimer, and IL-6 were more likely to worsen liver function in COVID-19 patients following HBV coinfection. Two weeks after the onset of symptoms, serum LDH levels were increased only in patients with COVID-19 and HBV coinfection compared with patients without HBV coinfection[80]. The conditions of four chronic HBV-infected patients were further aggravated after SARS-CoV-2 coinfection, as presented by progressive jaundice elevation, coagulation disorders, and ascites, and developed acute-on-chronic liver failure[78]. These findings suggest that SARS-CoV-2 and HBV coinfection is related to disease severity and poor clinical presentation, and affects liver function tests and that HBV inactive carriers have more severe liver damage. This may be due to the altered immune status caused by SARS-CoV-2 coinfection. Other possible reasons include inactive HBV carriers, who are more prone to HBV reactivation and liver injury with ALT flares caused by SARS-CoV-2 coinfection, and these patients have increased sensitivity to hepatotoxic antiviral drugs used for COVID-19 treatment[80].

There are opposing opinions that chronic HBV coinfection is not a predictive factor for the disease severity or poor prognosis of COVID-19. A study using a prognostic model with a nomogram indicated that HBV infection was not linked to the mortality risk of COVID-19[81]. Several studies have supported

this hypothesis. A cohort study of 326 confirmed COVID-19 patients shows that 20 (6.1%) had HBV coinfection, and the outcomes of the 20 patients (including rates of severe/critically ill, mortality, and discharged and hospital stays) showed no difference from those of patients without coinfection, and HBV coinfection did not increase the degree of liver injury[82]. This is consistent with the results of a previous review indicating that the degree of hepatic damage in patients with HBV and SARS-CoV-2 coinfection is not significantly different from that in patients with SARS-CoV-2 infection alone[83]. Therefore, whether or not HBV infection affects the clinical manifestations and outcomes of COVID-19, and how, requires further study.

Metabolic dysfunction-associated fatty liver disease and COVID-19

Metabolic dysfunction-associated fatty liver disease (MAFLD), previously defined as NAFLD, has been reported to be associated with increased ICU admissions and the need for mechanical ventilation after infection with SARS-CoV-2. However, it was not a predictive factor for death in COVID-19[18]. MAFLD is the most frequently occurring chronic disease in the world, with a common incidence of 30%, and is regarded as a liver performance of the metabolic syndrome. There is a distinct lack of research regarding the history of liver disease in COVID-19 patients and the connection between MAFLD and COVID-19[84]. A meta-analysis of COVID-19 comorbidities showed upregulation of ACE2 receptor expression in NAFLD[85]. A cohort study of 202 consecutive COVID-19 patients showed that patients with NAFLD had prolonged virus-shedding time and increased risk of disease severity compared to non-NAFLD patients[86]. In addition, a previous study has shown that younger patients (age < 60 years) with MAFLD have increased disease severity, possibly due to the higher incidence of cytokine storm caused by MAFLD in younger patients[87]. This is consistent with an alternative multicenter study that compared the risks of developing severe COVID-19 between younger and older patients with MAFLD. Results showed that 55.9% of the younger and 24% of the elderly developed severe COVID-19 (P = 0.01)[88], suggesting that younger MAFLD patients have an higher risk of developing severe COVID-19, while MAFLD in older patients appears to have little association with disease severity.

The mechanism underlying age-related disease severity in COVID-19 remains unclear. Possible explanations include: (1) Younger MAFLD patients are more likely to exhibit liver and systemic immune responses, and thereby a cytokine storm after SARS-CoV-2 infection; (2) older MAFLD patients usually have a higher burden of comorbidities which may harm COVID-19 and mask the link between MAFLD and COVID-19 severity[88]; (3) cytokine storm has been shown to increase in obese patients, especially in those with preexisting MAFLD[89]; (4) BMI is much higher in patients with NAFLD than in those without[90]; (5) MAFLD is also linked to abnormal levels of aminotransferases and GGT[89]; and (6) the connection between MAFLD and the severity of COVID-19 may be related to the release of pro-inflammatory mediators, such as, tumor necrosis factor-alpha and IL-6[91]. Notably, one study reported that the prognosis of MAFLD is closely related to the degree of fibrosis, and thus could affect the outcomes of COVID-19[63]. When MAFLD is accompanied by severe advanced liver fibrosis, it may exacerbate the cytokine storm caused by the virus, thus the release of proinflammatory cytokines leads to more severe COVID-19. In conclusion, unhealthy lifestyles contribute to the high morbidity of MAFLD and high risk of severe COVID-19.

Alcoholic liver disease and COVID-19

A previous study revealed an unprecedented increase in both ALD and liver transplant rates during the spread of COVID-19[92]. Therefore, more research focus should be placed on ALD, in relation to the COVID-19 pandemic. It was reported by the Centers for Disease Control and Prevention in the US that the mortality due to ALD has increased rapidly during the COVID-19 pandemic. One possible reason for this is the high consumption of alcohol and the increase in alcohol-related comorbidities before and during the COVID-19 pandemic. Other possible reasons include increased obesity/metabolic syndrome and NAFLD, which also contribute to the risk of ALD development. In addition, a study found that females and young adults showed the highest relative increases in ALD-related mortality, while older adults over the age of 85 years presented a smaller increase in overall female mortality and even a decrease in male mortality[93]. A comprehensive study of 978 CLD patients with SARS-CoV-2 infection from 21 institutions in the United States revealed a novel association between ALD and poor survival or COVID-19 related mortality. It has been confirmed that patients with preexisting ALD may be more susceptible to contracting COVID-19 due to immune system dysregulation. ALD is associated with a sterile inflammatory state caused by an injury-related molecular pattern, resulting in the spread of pro-inflammatory cytokines throughout the body. In addition, SARS-CoV-2-induced cytokine storm may exacerbate inflammation in patients with ALD, leading to worse clinical outcomes[94]. In a report on the trends in alcohol consumption and liver disease before and after COVID-19, more than a third of patients with CLD and 50% with ALD consumed alcohol daily, suggesting that high alcohol consumption may indicate a relationship with poor prognosis of COVID-19. There are many possible reasons why alcohol abuse leads to poor outcomes. Alcohol abuse and related liver disease can disrupt the innate and acquired immune systems due to impaired immune cell function and survival, which participate in defending against viral infections. Another reason may be that patients with chronic alcohol consumption are more likely to develop ARDS, thus leading to a worse outcome[95].

Liver transplant and COVID-19

Liver transplant recipients are more likely to develop severe COVID-19, due to chronic immunosuppression and related comorbidities, which may lead to mortality[96]. A systematic search of 15 studies consisting of 223 Liver transplant patients, among which 77.7% required hospitalization, found that 36% experienced more severe disease, with a higher mortality rate observed in the cohort compared with approximately 1%-4% in the general population. In addition, liver transplant recipients often present with fever and dyspnea which are similar to common COVID-19 manifestations; however, liver transplant recipients are more likely to have concurrent diarrhea symptoms. Furthermore, older liver transplant recipients (aged > 60 years) with diabetes are at an increased risk of mortality[72]. Another study compared 151 Liver transplant patients (68% male, median age of 60 years) with 627 non-transplanted patients (52% male, median age of 73 years), showing that groups of liver transplant patients tended to have ICU admission (28% *vs* 8%) and needed treatment with invasive ventilation (20% *vs* 5%), while mortality was lower in the liver transplant cohort than in the non-transplant cohort (19% *vs* 27%). Several statistical analyses have shown age-related mortality among liver transplant recipients[18]. Similarly, the first nationwide study compared the morbidity and outcomes of 111 Liver transplant patients infected with SARS-CoV-2 with those of the general population, matched for age and sex[97]. Liver transplant recipients have a doubled risk of developing COVID-19, but have a lower standardized mortality rate compared to the general population. They conclude that chronic immunosuppression and increased comorbidity make the liver transplant recipients more likely to suffer from the infection of the virus and develop to COVID-19, whereas chronic immunosuppression can resist the most severe forms of COVID-19 and play a protective role against the virus, resulting in reduced mortality rates.

DISCUSSION

COVID-19 caused by SARS-CoV-2 infection is a worldwide epidemic, which has had catastrophic impacts on healthcare systems and social management. Although SARS-CoV-2 mainly affects the respiratory tract, current studies have also focused on multi-organ injury particularly liver injury, with liver test abnormalities identified as an important extrapulmonary manifestation related to SARS-CoV-2 infection[98]. As liver dysfunction is associated with disease severity and poor clinical presentation, understanding the pathogenesis of liver injury and its associated risk factors is extremely important for the prognosis and treatment of the disease. This review briefly summarizes the onset, mechanism, risk factors, susceptible groups, and correlation between liver injury and the clinical outcomes of COVID-19.

According to previous studies, hypoalbuminemia and elevated AST and ALT levels are frequently observed in severe COVID-19 cases, indicating abnormal liver function. These elevations are usually accompanied by elevated LDH. GGT levels were also generally elevated, but ALP elevation was not evident in most studies. Although it is debated whether the pattern of liver injury is hepatocellular or cholestatic, the vast majority of studies support the hepatocellular injury. However, because of the high expression of ACE2 receptors in cholangiocytes, the hypothesis that liver injury results from the direct cytopathic effect of SARS-CoV-2 on hepatocytes seems unconvincing. Liver injuries likely reflect the severity of the disease. Liver histopathological studies predominantly showed moderate microvascular steatosis and slight lobular activity and inflammatory infiltration of the portal area in the biopsy and autopsy of COVID-19 cases. However, the exact mechanisms of liver injury in COVID-19 are still unclear, perhaps more cases are due to indirect liver injury.

Predisposing risk factors for liver damage in COVID-19 include male sex, older age, other comorbidities, and underlying CLDs. Men are more likely to develop liver injury than women because of the stronger innate immune system in men and estrogen protection in women. Patients aged > 65 years appear to exhibit a progressive increase in the risk of developing severe disease. Obesity is also a contributing factor to severe COVID-19, because dysregulated hepatic innate immunity, adaptive immune response, and pro-inflammatory state are usually present in fatty cases, which may aggravate the cytokine storms. Cytokine storms can be enhanced in patients with preexisting MAFLD. The contributions of other preexisting CLDs (including cirrhosis, ALD and chronic hepatitis B) to the progression of disease severity and liver injury in COVID-19 remain controversial. Most studies have emphasized that CLDs are more susceptible to severe COVID-19 due to immune disorders, and CLDs are often associated with abnormal liver function tests. However, CLD is not a predictor of mortality. Liver transplant recipients have many potential risk factors for developing worse outcomes in COVID-19, but it appears that chronic immunosuppression can protect COVID-19 patients from developing severe disease and thus reduce the mortality. Older liver transplant recipients (aged > 60 years) with diabetes are at increased risk of mortality. The exact link between CLD and COVID-19 is unclear, and whether CLD aggravates liver dysfunction and worsens the clinical outcomes of COVID-19 remains ambiguous.

CONCLUSION

Liver injury is frequent in patients with COVID-19 and abnormal liver function tests are more often observed in severe cases. Risk factors and populations for liver dysfunction in COVID-19 patients include male sex, older age, underlying comorbidities (particularly diabetes, hypertension, and obesity) and preexisting liver diseases (CLD and liver transplant). The mechanisms underlying liver injury in COVID-19 patients remain unclear, which are more likely due to the direct effect of the virus on hepatocytes or the biliary epithelium, and the indirect effects of excessive inflammation, drugs, and ischemia/hypoxia syndromes. Collectively, liver injury in COVID-19 patients is associated with increased disease severity and requires additional attention and effective treatments. In addition, targeted therapy is needed for patients with preexisting liver diseases, especially older patients with comorbidities. Further research is needed to determine whether the underlying disease coexists with COVID-19 to cause severe diseases that causes liver damage, or whether the underlying conditions directly contribute to liver damage. Attention should also be paid to DILI during the treatment of COVID-19, especially the conventional and new drugs used for specific groups. The information discussed in this review may aid in establishing recommendations and guidance for the treatment of liver injury in COVID-19 to reduce liver burden.

Table 1 Abnormal liver function with existing liver disease and effects of COVID-19

Ref.	No. of patients included	No. of patients with preexisting liver disease	Liver function markers	Impact on disease
Cirrhosis				
Iavarone *et al* [76], 2020	399	50	Bilirubin and ALT significantly increased, while albumin significantly decreased	Elevated transaminases may have an adverse impact on the process of cirrhosis and the 30-d mortality rate was higher in those patients who had impaired liver function
Marjot *et al* [77], 2021	745	386	-	SARS-CoV-2 infection in patients with cirrhosis appears to be associated with high rates of acute hepatic decompensation (46%) and patients would have a 2-fold increased rate of mortality
Hepatitis B				
Zou *et al*[78], 2021	105	105	Elevated levels of ALT (22, 20.95%), AST (29, 27.62%), total bilirubin (7, 6.67%), GST (7, 6.67%), and ALP (1, 0.95%)	Liver injury in patients with SARS-CoV-2 and chronic HBV co-infection was associated with disease severity
Chen *et al* [82], 2020	376	20	No significant increase	Coinfection with SARS-CoV-2 and HBV slightly affected liver function and had no effect on COVID-19 outcomes
MAFLD				
Zhou *et al* [88], 2020	327	93	-	Younger COVID-19 patients (aged < 60 years) with MAFLD have a more than 2-fold higher prevalence of severe COVID-19 while MAFLD in older patients appears to have no relation to the severity of the disease
Tripon *et al* [89], 2022	719	445	-	SARS-CoV-2-induced cytokine storm can be enhanced in patients with a preexisting liver disease like NAFLD
ALD				
Kim *et al*[94], 2021	367	94	-	Patients with ALD were at higher risk of contracting COVID-19 due to their immune system dysregulation and SARS-CoV-2-induced cytokine storm may exacerbate inflammation in ALD patients
Liver transplant				
Fraser *et al* [72], 2020	223	223	-	In liver transplant recipients with COVID-19, 77.7% required hospitalization, 36% experienced more severe disease, and the mortality rate observed in the cohort was 19.3%
Colmenero *et al*[97], 2021	111	111	-	In liver transplant patients, chronic immune-suppression increases the risk of developing COVID-19 but it could reduce disease severity and the mortality

SARS-CoV-2: severe acute respiratory syndrome coronavirus 2; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GST: Gamma-glutamyl transpeptidase; MAFLD: Metabolic dysfunction-associated fatty liver disease; ALD: Alcoholic liver disease.

FOOTNOTES

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MINIREVIEWS

Obstructive and secretory complications of diverting ileostomy

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Abstract

This review aimed to highlight the etiology, diagnosis, treatment, and prevention of obstructive and secretory complications associated with diverting ileostomy (DI). Obstructive complications at the stoma site are termed stoma outlet obstruction (SOO) or stoma-related obstruction (SRO). The incidence of SOO/SRO is 5.4%-27.3%, and the risk factors are multifactorial; however, the configuration of the stoma limb and the thickness of the rectus abdominis muscle (RAM) may be of particular concern. Trans-stomal tube decompression is initially attempted with a success rate of 33%-86%. A thick RAM may carry the risk of recurrence. Surgical refinement, including a wider incision of the anterior sheath and adequate stoma limb length, avoids tension and immobility and may decrease SOO/SRO. Secretory complications of DI are termed high output stoma (HOS). Persistent HOS lead to water and sodium depletion, and secondary hyperaldosteronism, resulting in electrolyte imbalances, such as hypomagnesemia. The incidence of HOS is 14%-24%, with an output of 1000-2000 mL/d lasting up to three days. Treatment of HOS is commenced after excluding postoperative complications or enteritis and includes fluid intake restriction, antimotility and antisecretory drug therapies, and magnesium supplementation. Intensive monitoring and surveillance programs have been successful in decreasing readmissions for dehydration.

Key Words: Small bowel obstruction; Stoma outlet obstruction; Stoma-related obstruction; High output stoma; High output syndrome; Dehydration

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Core Tip: This review highlights the etiology, diagnosis, treatment, and prevention of obstructive and secretory complications associated with diverting ileostomy (DI). Obstructive complications at the stoma site (stoma outlet obstruction/stoma-related obstruction, SOO/SRO) affect 5.4%-27.3% of patients with DI. Trans-stomal tube decompression is effective in most cases. Surgical refinement is important for reducing SOO/SRO. Secretory complications (high output stoma, HOS) lead to water and sodium depletion and secondary hyperaldosteronism with electrolyte imbalances. The incidence of HOS is 14%-24%, with an output of 1000-2000 mL/d. HOS treatment includes fluid intake restriction, antimotility and antisecretory drug therapies, and magnesium supplementation. Intensive monitoring and surveillance programs may decrease the readmission rates for dehydration.

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INTRODUCTION

Diverting ileostomy (DI) is often performed in patients undergoing low anterior resection for rectal cancer or restorative proctocolectomy with ileal-pouch anal anastomosis (IPAA) for ulcerative colitis. The purpose of DI creation is to protect the anastomosis from leakage and mitigate the severity of symptoms relative to anastomotic complications. However, the efficacy of DI is solely limited to a significant decrease in anastomotic leakage, and no other advantages may be found in the short term[1,2]. Moreover, stoma formation significantly increases the risk of small-bowel obstruction, postoperative ileus, dehydration, and electrolyte imbalance[2-4]. Some of these complications are specific to DI and can be classified into "obstructive" and "secretory" complications. The risk factors for developing these complications have been widely discussed, but the fundamental mechanisms and management of these complications are not fully understood. This review aimed to highlight the etiology, diagnosis, treatment, and prevention of obstructive and secretory complications associated with DI.

METHODOLOGY

An electronic English literature search was performed on PubMed/MEDLINE database from the inception to September 15, 2022. The search items included "diverting ileostomy" or "covering ileostomy", "small bowel obstruction", "stoma outlet obstruction" (SOO) or "outlet obstruction", "stoma-related obstruction" (SRO), "high output stoma" (HOS) or "high output syndrome", and "dehydration". Inclusion criteria for the article type were systematic review and meta-analyses, randomized controlled studies, retrospective observational studies, and narrative reviews for nursing aspects. Case reports were not included.

OBSTRUCTIVE COMPLICATIONS

Etiology

Small bowel obstruction (SBO) is one of the most common complications associated with colorectal surgery. The increased incidence of SBO when DI is created at the time of the initial surgery is of particular concern[4-8]. Since DI is brought up extracorporeally through the abdominal wall by splitting the rectus abdominis muscle (RAM), scar formation or tissue inflammation at the anterior rectus sheath (where the incision is made) may lead to stenosis of the stoma opening (outlet). Furthermore, the stoma outlet is physiologically vulnerable to the risk of obstruction, and its underlying mechanisms can be explained by the following speculations. First, the stoma is edematous, and the bowel lumen tends to be narrower in the early postoperative period. Second, the intraluminal pressure of the small bowel is lower than that of the colon, suggesting relative stenosis at the RAM level[9]. Third, if a high volume of bowel content flows into the lumen, the stoma outlet may have a change in caliber, leading to relative narrowing[10].

Obstructive complications at the stoma site have been termed SOO and SRO in the literature. Okita *et al*[11] proposed a definition of SBO at the stoma site that has the following criteria: (1) Radiographically confirmed dilatation of the oral stoma limb; (2) Increase in stomal output and relief of symptoms after trans-stomal tube decompression; and (3) Exclusion of SBO other than that at the stoma site. Some of the subsequent reports have followed these criteria for diagnosis[10,12-14], while others have used more

simplified criteria, such as SBO at the stoma site with radiographic confirmation excluding paralytic ileus[15-17].

Risk factors

SOO/SRO occurs in 5.4%-27.3% of patients with DI[10-18]. Risk factors are classified into the following categories: patient characteristics[10,11,13,16,19] (young age, low body mass index, thick subcutaneous fat, thick RAM, and long distance between the superior mesenteric artery root and the bottom of the external anal sphincter), disease[15] (ulcerative colitis), surgical factors[12,14,20-22] (laparoscopic surgery, rotation of stoma limb, IPAA, two-stage surgery for ulcerative colitis, and short distance from the anastomosis to the stoma site), and stoma functions[10,17] (high output from stoma). The risk factors are summarized in Table 1.

Young age (less than 16 years old) and low body mass index (less than 21 kg/m^2) in patients with ulcerative colitis were reported as risk factors by Okita *et al*[11]. The mechanisms and interpretation of the results were not clearly shown, although they assumed that the small abdominal cavity or small amount of mesenteric fat in these individuals allowed volvulus or kinking. A contradictory result was reported by Tamura *et al*[16], who found that increased subcutaneous fat [vertical distance of 20 mm or more at the stoma site marking on computed tomography (CT)] in obese patients was a significant predictor of SOO/SRO. However, the different pathologies in the study population (inflammatory disease *vs* colorectal cancer) make this comparison difficult. The authors assumed that the tension and twisting in the loop stoma might have been caused by the minimum size of stoma apertures at the skin and tight with a narrow subcutaneous cavity in obese patients, and thus surgeons should be aware of it. Mori *et al*[19] reported that SOO/SRO was more common in patients with a long distance between the superior mesenteric artery root and the bottom of the external anal sphincter (height-adjusted, 191 mm/m on CT), suggesting that the mesentery may be under tension. The authors encouraged surgeons to reduce tension in the mesentery using all applicable surgical techniques. Thick RAM at the stoma passage (vertical length of 10 mm or more on CT) has been shown to increase the risk of SOO/SRO[10,13], and it is speculated that the intraluminal pressure of the stoma may be overwhelmed by the increased pressure of the thick RAM[10]. The authors assumed that surgeons need to create a wider split of the RAM for these patients.

As a disease-related factor, ulcerative colitis has been reported as an independent risk factor for SOO/SRO, but the specific reason remains unclear[15]. It is speculated that the risk may not be due to the disease itself; rather, it refers to the surgical procedure of total proctocolectomy with IPAA with DI (also known as the first of the two-stage surgery)[12,20]. If the length of the diverted bowel (between the stoma and pouch) is too short, strong tension in the mesentery may occur, and it is difficult to revert if bowel twisting or mesenteric torsion occurs. If the length is too long, bowel twisting, kinking, or angulation may occur; however, a spontaneous resolution is expected in such cases[12,18]. Several studies have shown an increased risk of SOO/SRO in laparoscopic surgery[18,22]. Laparoscopic surgery essentially reduces adhesion formation and may induce torsion of the mesentery or bowel as a result of increased bowel mobility. Regarding rotation, the risk of SOO/SRO remains controversial. Forced stoma rotation by 180-degree with IPAA significantly increases the incidence of SBO; bowel kinking at or below the fascia is presumably responsible for the obstruction[21]. In contrast, recent reports have shown that stoma rotation did not increase the incidence of SOO/SRO[11,12,15,18,20]. The reason for the SOO/SRO associated with high output from the stoma has already been described in the *Etiology* section.

Diagnosis

To examine whether the outlet is stenotic or obstructive, clinicians can simply insert their fingers into the stoma[23]. An abdominal CT scan or contrast enema study through the stoma may be useful for precise diagnosis, as they can demonstrate the location responsible for SOO/SRO and exclude other sites of SBO (Figures 1 and 2). Other pathologies causing similar symptoms, such as paralytic ileus, enteritis, volvulus, internal hernia, and parastomal hernia, should also be excluded.

Treatment

In cases of SBO, fasting, bowel rest, and intravenous fluid administration are fundamental recommendations. When nausea, vomiting, or abdominal bloating/distention is present, a nasogastric tube may be placed. Although uncommon in Western societies, long nasointestinal tubes may be indicated to achieve quicker and more effective intraluminal decompression[11,24,25]. Trans-stomal tube decompression is attempted as an initial treatment for SOO/SRO. The success rate of local management using trans-stomal tubes ranges from 33%-86%[11,12,15,17]. Redo treatment may be indicated in cases of recurrent obstructions. It has been shown that thick RAM carries the risk of recurrent obstruction[17]. Importantly, adverse events related to trans-stomal tube decompression have also been reported. Bowel injury occurred in 3.7% of the intubated patients requiring emergency surgery. Stoma closure is the ultimate solution for SOO/SRO in patients for whom non-surgical, conservative management, or repeated tube decompressions were not successful.

Table 1 Risk factors of obstructive complication of diverting ileostomy

Risk factors
Patient characteristics
Young age (less than 16 yr old)[11]
Low body mass index (less than 21 kg/m^2)[11]
Thick subcutaneous fat at the stoma marking site (20 mm or more) [16]
Thick rectus abdominis muscle at the stoma passage (10 mm or more)[10,13]
Long distance between the superior mesenteric artery root and the bottom of the external anal sphincter (height-adjusted, 191 mm/m)[19]
Disease
Ulcerative colitis (compared with colorectal cancer)[15]
Surgical factors
Laparoscopic surgery (compared with open surgery)[18,22]
Rotation of stoma limb (180-degree rotation, the oral limb situated on the caudal side)[21]
Ileal-pouch anal anastomosis (compared with low anterior resection or intersphincteric resection)[12]
Two-stage surgery for ulcerative colitis[20]
Short distance from the ileal pouch to the stoma site (< 30 cm)[12,18]
Stoma functions
High output from stoma (2000 mL or more per day)[10,17]

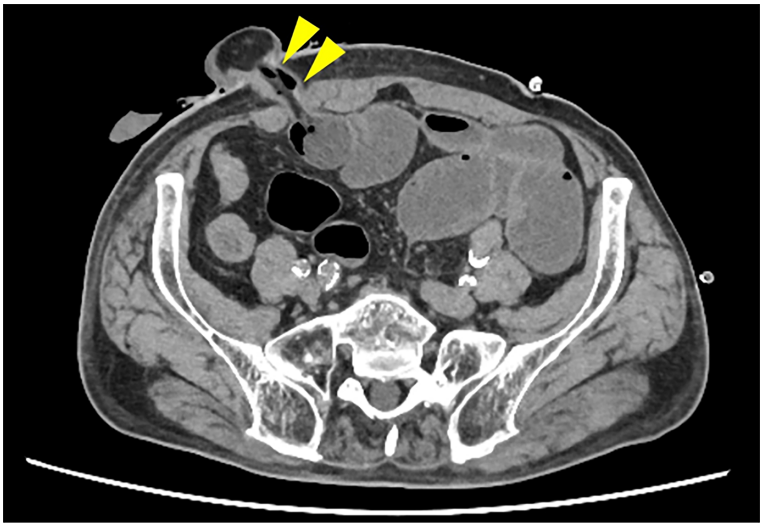

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Figure 1 Computed tomography shows stenosis at the ileostomy site (arrowheads).

Prevention

Some of the risk factors for SOO/SRO, such as patient, disease, anatomical, and stoma function, may not be preventable, and surgical refinement must be fully considered. When creating DI, the anterior sheath is adequately incised, and the RAM is split and dilated to accommodate the stoma limbs. In the case of thick RAM, creating the tunnel by the conventional "two finger breadths rule" may not be sufficient; thus, wider dissection is necessary[13]. Moreover, care must be taken in laparoscopic surgery because the stoma is constructed under the effect of pneumoperitoneum with muscle relaxation[14]. In the case of IPAA, the distance between the ileal pouch and anastomosis must be longer than 30 cm to mitigate the risk of tension in the mesentery[12,18]. Rotation of the stoma limb is carefully performed if necessary. Although they did not advocate stoma rotation, Takehara *et al*[14] proposed that the oral stoma limb should be placed on the cranial side to avoid gravitational compression by the anal stoma limb. Some authors have recommended intra-abdominal suture fixation between the stoma limb and abdominal wall[26,27]; however, it might also carry the risk of immobility, tension, and fecal impaction

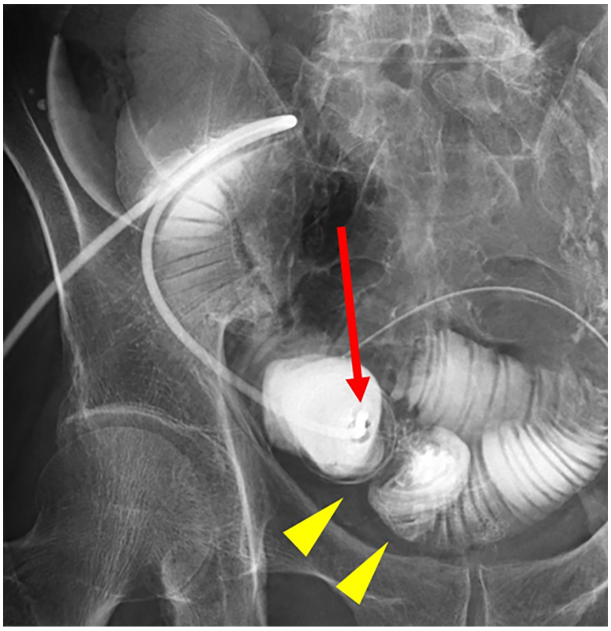

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Figure 2 Contrast enema study shows a decompression tube inserted at the stenotic stomal opening (arrow) and dilated small bowel proximal to the stoma site (arrowheads).

in the stoma.

SECRETORY COMPLICATION (HIGH OUTPUT)

Etiology

In healthy adults, approximately 1500 mL of intestinal fluid enters the colon from the ileum[28]. Theoretically, the same amount of fluid is drained from the newly established ileostomy; however, the normal output of ileostomy has been proposed as 600-1200 mL per day and may decrease over time[29-31]. This reduction in the quantity is called "ileostomy adaptation," which suggests a compensatory increase in anti-diuretic hormones such as renin and aldosterone[28]. The consistency of the ileostomy output is generally watery when created and thickens in the next 2-3 mo[29]. Stool consistency, amount, and ileostomy output may be altered by the patient's body weight, disease for the index surgery, liquid and food intake, and volume of gastrointestinal secretions[29,32].

Secretory complications of DI occur when the output exceeds the aforementioned normal limit. It is commonly termed "HOS" in the literature. This term may be considered similar to "dehydration'. Dehydration has been shown to be a cause of readmission in 9.3%-43% of the patients after ileostomy creation without preventive protocols[33-35]. The most recent meta-analysis showed that the pooled incidence of readmission due to dehydration was 6% regardless of HOS prevention and was accompanied by increased medical costs[36]. Moreover, HOS results in electrolyte imbalance and acute kidney injury in the early postoperative period and may lead to malnutrition and chronic renal impairment in the long term[30,37]. The prevalence of *Clostridium difficile* infection in the development of postoperative diarrhea/HOS was investigated, but it was found that the infection rate was low (1.6%) and that the patient outcomes were not affected by the infection[38]. Contradictory results have also been reported[39].

The ileostomy output includes large amounts of sodium (85-180 mmol/L per day)[40]. Persistent HOS can lead to sodium depletion, dehydration, and secondary hyperaldosteronism, which may cause sequential hypokalemia and hypomagnesemia, which are the main features of electrolyte imbalances. As the ileum and colon are the main sites of magnesium absorption, ileostomy patients may be susceptible to hypomagnesemia. Other reasons for hypomagnesemia include free fatty acid malabsorption and the use of proton pump inhibitors[31]. Baker *et al*[29] reported that nearly half of the patients with HOS exhibit hypomagnesemia.

Definitions

The incidence of HOS varies from 14% to 24%[29,41-43] with various definitions. The majority of previous reports have defined using specific values of output volume with a timeframe, such as 2000 mL per day[38,44,45], 1500 mL per day for two days[41], 1000 mL per day for three days[42,46], and

2000 mL per day for three days[29,43]. Some authors have used a combination of output volume with laboratory findings of renal impairment and/or physical signs of dehydration[36,47].

HOS can occur in the early or late postoperative periods[29]. In a study, early HOS occurs within three weeks after stoma creation and resolves spontaneously in 49% of patients with HOS, while 7% receive persistent treatment. Late HOS occurs more than three weeks after stoma creation and resolves spontaneously in 15% of patients with HOS, while 47% receive persistent treatment. In that study, the diagnosis of cancer, followed by perforation and short bowel (less than 200 cm), correlated with the incidence of early HOS, whereas inflammatory bowel and obstruction were deemed to have an impact on late HOS[29]. Therefore, secretory complications of DI are an ongoing problem after stoma creation, and patients are always at risk, even after hospital discharge. This issue has become more relevant in the era of enhanced recovery after surgery.

Causes and risk factors

The causes of HOS are multifactorial, including factors associated with patients[42,48], disease[29,42,43], anatomy[29], surgical procedure[29,42,43,48], medication[29,33], nutrition[29], enteritis/metabolism[29,39] and those related to postoperative complications[29,44,49]. Older age was identified as an independent risk factor of HOS in two studies: however, the cut-off values were not shown because the comparisons were made without dichotomization in their studies[42,48]. The reasons were not specified in either of the studies. Assaf *et al*[48] also showed that higher American Society of Anesthesiologists-physical status and elevated creatine levels were independently associated with HOS. Again, the cut-off values were not shown for the same reason as the older age. The authors assumed that impaired kidney functions resulted in less adaptation to fluids and electrolyte loss in these groups of patients.

Takeda *et al*[43] found that diabetes was one of the risk factors for HOS, with the following explanations. Diabetes may cause autonomic nervous system impairment that decreases the motor function of the bowel, followed by abnormal proliferation of intestinal bacteria and an augmented intestinal pressure with increased gas proliferation. Inflammatory bowel diseases, particularly Crohn's disease, have been shown to be significantly associated with HOS[29,42,43]. Crohn's disease presents an impaired intestinal permeability/barrier increased with altered gut microbiota and inflammatory tissue damage[42]. These factors can lead to HOS. Shortening of the small bowel with a more proximal ileostomy can also lead to HOS, and this may explain the reason why some surgical procedures (right-side colectomy, separate ileostomy, small bowel resection, and IPAA) were independent risk factors of HOS. Takeda *et al*[43] proposed another reason for HOS in patients who underwent IPAA: Inhibition of lipids absorption leads to hydroxylation or desaturation of unabsorbed long-chain acids, which triggers intestinal fluid and electrolyte secretion. Open surgery was significantly associated with HOS compared to laparoscopic surgery[42,48]. The possible explanation is that open surgery itself may not be the direct cause but patients undergoing more complex surgery with open laparotomy[48]. A short bowel (less than 200 cm) frequently causes HOS[29], but this may not be applicable in DI formation, where a stoma is created at the distal ileum.

Perioperative medication is an important underlying pathology for the development of HOS. The use of diuretics may easily result in dehydration because the body fluid balance is vulnerable in patients with ileostomy, even without any medications[33]. Administration of prokinetic drugs (*e.g.*, metoclopramide) induces HOS; therefore, care must be taken when the patients suffer from nausea/vomiting with gastric stasis[31]. Sudden withdrawal of opiates and steroids induces reactive intestinal secretion[29-31]. Diarrhea with dehydration is commonly seen in patients who receive chemotherapy consisting of cytotoxic agents.

Regarding nutritional factors, intake of hypotonic fluids such as water, tea, coffee, fruit juice, and alcohol precipitates dehydration, and thus it is generally restricted or avoided[29-31]. Enteric infections, including *Clostridium difficile* or *Salmonella*, typically present with acute and severe diarrhea[29,50]. Chronic diarrhea may be caused by bacterial overgrowth from diverticula or blind loop fermentation[29,50].

Postoperative complications may induce secondary HOS, including prolonged ileus[44] (presented with nausea, vomiting, intolerance to oral feeding, abdominal distension, or failure to pass flatus or bowel movements within postoperative 7 d), SBO[29], SOO/SRO[49], or intra-abdominal sepsis/deep surgical site infection[29,49]. These postoperative complications must be excluded clinically and radiologically when suspicious of HOS. The details of the causes and risk factors of HOS are summarized in Table 2.

Diagnosis

The key clinical symptoms of HOS are due to the loss of water and sodium and include thirst, cramps, muscle weakness, and faintness[31,50]. Patients may also present with loss of appetite, rapid weight loss, fall in postural blood pressure, or a decrease in urinary output[31]. The stomal output volume is measured every 8 h to facilitate early recognition of HOS. The stoma color and stool consistency should be inspected. Because stenosis or obstruction at the stoma outlet is one of the causes of HOS, a digital examination of the stoma is suitable for diagnosis. Laboratory findings may include elevated serum urea/creatinine ratio, hyponatremia, hypokalemia, and hypomagnesemia. A decrease in urinary sodium (less than 10 mmol/L) reflects sodium depletion more accurately than serum sodium level[31,

Table 2 Causes and risk factors of high output stoma

Causes and risk factors of high output stoma
Patient
[1]Older age[42,48]
[1]Higher ASA-PS[48]
[1]Elevated baseline creatine[48]
Disease
[1]Diabetes[43]
Inflammatory bowel disease (*i.e.*, ulcerative disease, Crohn's disease)[29,42,43]
Anatomy
Short bowel (less than 200 cm)[29]
Surgical procedure
Open surgery (*vs* laparoscopic surgery)[42,48]
Total proctocolectomy (with ileal-pouch anal anastomosis)[42]
Right-side colectomy[42]
Separate ileostomy[42]
Small bowel resection[29,42,43]
Medication
Preoperative use of diuretics[31,33]
Prokinetic drugs (*i.e.*, metoclopramide)[29,31]
Sudden withdrawal of corticosteroids or opiates[29,31]
Postoperative adjuvant chemotherapy[29]
Nutrition
Hypotonic liquids (low sodium): water, tea, coffee, fruit juice, alcohol[29,30,31]
Enteritis/metabolism
Clostridium difficile infection[29,50]
Salmonella infection[29,50]
Bacterial overgrowth from diverticula or blind loop fermentation[29,50]
Relative to postoperative complications
Postoperative ileus (symptoms of nausea, vomiting, intolerance to oral feeding, abdominal distension, or failure to pass flatus or bowel movements within postoperative 7 d)[44]
Intra-abdominal sepsis (pelvic sepsis, organ/space infection)[29,49]
Small bowel obstruction[29]
Stoma outlet obstruction[49]

[1]No specific values or definitions were shown in the referenced articles. ASA-PS: American Society of Anesthesiologists-physical status.

50]. Long-lasting HOS may decrease the absorption of vitamin B12 and folic acid and increase the incidence of renal calculi and gallstones[40].

Treatment

Exclusion of possible causes: As shown in Table 2, HOS occurs secondary to the underlying intra-abdominal complications. CT is useful for identifying these factors, and treatment of the identified cause must be prioritized. The administration or cessation of certain drugs is responsible for HOS and is corrected accordingly after the diagnosis. Enteritis induced by *Clostridium difficile* or *Salmonella* is excluded by taking stool cultures from the output.

Restriction of fluid intake: Since patients with dehydration complain of thirst in the early phase of HOS, increasing fluid intake may be inappropriately advised to relieve symptoms[30]. Consumption of excessive hypotonic fluids (less than 90 mmol/L of sodium) leads to worsening symptoms with sodium depletion caused by a net efflux from the plasma into the bowel lumen[29,50]. Hypertonic fluids containing glucose may also lead to increased stomal output[50]. Previous reports have suggested that hyper- and hypotonic fluid intake must be restricted to 0.5-1.0 mL per day[29,31,50]. Oral intake may not be possible when presenting with nausea/vomiting, and intravenous fluid rehydration containing 100-150 mmol/L of sodium (*e.g.*, normal saline) is necessary to avoid acute kidney injury[31]. If oral intake is possible, it is recommended to sip 1 L or more of a glucose-saline solution containing at least 90 mmol/L sodium in a small quantity at intervals[31,50].

Drug therapies: Antimotility drugs include loperamide and codeine phosphate. Both drugs act against intestinal motility, and it has been shown that the ileotomy output reduces by 20%-30%[31]. Loperamide is preferred over codeine phosphate because it is not addictive. Loperamide can be prescribed at 4 mg/d to 16 mg/d, while codeine phosphate can be prescribed at 15 mg/d to 60 mg/d, and the effect becomes greater if both are taken together[31,41,50].

Antisecretory drugs include H_2 antagonists and proton pump inhibitors that reduce gastric acid secretion. The somatostatin analog, octreotide, reduces gastric and pancreaticobiliary secretions and delays gastric emptying and small-bowel transit. Antisecretory drugs may be used when the stomal output exceeds 2 L/d, but octreotide is not preferred because the proton pump is as effective as octreotide in reducing the stomal output[29,31,41].

Hypomagnesemia in patients with HOS is caused by the chelation of magnesium with unabsorbed fatty acids and increased magnesium secretion due to secondary hyperaldosteronism[50] and reduced absorption of fatty acids. Magnesium is preferably supplemented orally; however, it can be slowly administered intravenously to avoid a flushing sensation.

Stoma closure: HOS may persist in 5.0%-15% of the affected patients until the stoma is reversed[29,35]. Stoma closure is planned for patients who do not respond to non-surgical conservative treatment. In a study, patients with readmission due to dehydration underwent stoma closure earlier than those without readmission[33]. Ihnát *et al*[5] reported that 3.8% of patients with DI underwent acute surgery for HOS.

Prevention

Intensive monitoring and surveillance programs have been proposed recently. Most protocols consist of preoperative patient education, in-hospital monitoring and intervention protocols, post-discharge hospital visits, and surveillance using telephone or telemedicine platforms[51-54]. For example, Shaffer *et al*[54] proposed a pilot program for outpatient follow-ups using regular visits and telephone interviews. The triggers of intervention included tachycardia with > 100 beats/min, ileostomy output > 1200 mL/d, major changes in weight, fever, nausea, poor oral intake, dry mouth, and low urinary output. The response by the team included an assessment of the basic metabolic profile, intravenous hydration at home, and phone calls to the doctor's office. This program successfully reduced the incidence of readmission from 21% to 8.7% and the cost of readmission by more than 80%.

These intense programs have been mostly successful in decreasing readmissions for dehydration (15%-21% before intervention *vs* 5.0%-8.8% after intervention)[52-54]; however, a conflicting result has also been reported, where the program failed to decrease readmissions for dehydration (8.2% *vs* 5.9%) despite obtaining better phone follow-up and an increase in outpatient intravenous fluid in the intervention group[51].

LIMITATIONS

There are several limitations in this review. First, this is a narrative review without a systematic approach where a literature search was performed according to the authors' experiences using their preferred keywords of choice. Furthermore, inclusion for the selection was purely based on the authors' judgment considering their relevance to the topics. Second, the selected articles were mostly non-randomized, retrospective studies resulting in selection bias. Since the causes and risk factors of obstructive and secretory stoma complications are often multifactorial, it may be difficult to design prospective trials to investigate the efficacy of specific surgical or medical interventions. Instead, recent prospective studies have shown that postoperative monitoring and surveillance programs may facilitate early recognition and decrease the adverse events relative to the complications[51-54]. Third, the conclusions of the selected articles were based on their population-based analyses. The research findings may not be instantly applicable or comparable to the current practice of readers due to the differences in the definitions (diagnostic criteria), patient characteristics, disease, surgical settings, social background, and healthcare systems. The research findings must be carefully interpreted, considering these variables in the communities of interest.

CONCLUSION

Obstructive and secretory complications of DI occur both in the early and late postoperative periods and require intensive monitoring and intervention. Non-surgical conservative treatment is mostly effective; however, stoma closure may be considered in recurrent or refractory cases. To facilitate the diagnosis and treatment for patient safety, seamless communication and close collaboration in a multidisciplinary team are necessary.

Interestingly, obstructive and secretory complications may also occur simultaneously. However, the causative factors of these complex pathologies are various and inconsistent: the potential reason is that most evidence has been obtained by retrospective, observational studies with a small sample size. Further prospective randomized trials are needed to assess the efficacy of interventions or protocols to improve the outcomes of obstructive and secretory complications of ileostomy.

FOOTNOTES

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MINIREVIEWS

Role of the combination of biologics and/or small molecules in the treatment of patients with inflammatory bowel disease

Domingo Balderramo

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Abstract

Inflammatory bowel disease (IBD) is a group of chronic diseases that includes ulcerative colitis, Crohn's disease, and indeterminate colitis. Patients with IBD require prolonged treatment and high utilization of healthcare resources for proper management. The treatment of patients with IBD is focused on achieving therapeutic goals including clinical, biochemical, and endoscopic variables that result in improvement of the quality of life and prevention of disability. Advanced IBD treatment includes tumor necrosis factor inhibitors, integrin antagonist, antagonist of the p40 subunit of interleukin 12/23, and small molecule drugs. However, despite the multiple treatments available, about 40% of patients are refractory to therapy and present with persistent symptoms that have a great impact on their quality of life, with hospitalization and surgery being necessary in many cases. Dual therapy, a strategy sometimes applicable to refractory IBD patients, includes the combination of two biologics or a biologic in combination with a small molecule drug. There are two distinct scenarios in IBD patients in which this approach can be used: (1) Refractory active luminal disease without extraintestinal manifestations; and (2) patients with IBD in remission, but with active extraintestinal manifestations or immune-mediated inflammatory diseases. This review provides a summary of the results (clinical response and remission) of different combinations of advanced drugs in patients with IBD, both in adults and in the pediatric population. In addition, the safety profile of different combinations of dual therapy is analyzed. The use of newer combinations, including recently approved treatments, the application of new biomarkers and artificial intelligence, and clinical trials to establish effectiveness during long-term follow-up, are needed to establish new strategies for the use of advanced treatments in patients with refractory IBD.

Key Words: Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; Dual-therapy biologic therapy; Small molecule drugs; Clinical remission

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Core Tip: Patients with inflammatory bowel disease (IBD) require prolonged treatment and high utilization of healthcare resources. About 40% of patients are refractory to different treatments with an increase need for hospitalization and surgery. Dual therapy, a strategy applicable to refractory IBD patients, includes the combination of two biologics or a biologic in combination with a small molecule drug. There are two distinct scenarios in IBD therapy in which this approach can be used: (1) Refractory active luminal disease without extraintestinal manifestations; and (2) patients with IBD in remission, but with active extraintestinal manifestations or immune-mediated inflammatory diseases.

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INTRODUCTION

Inflammatory bowel disease (IBD) is a group of chronic diseases that includes ulcerative colitis (UC), Crohn's disease (CD), and indeterminate colitis. Patients with IBD require prolonged treatment and high utilization of healthcare resources for its proper management[1]. Medical treatment includes the use of so-called conventional drugs (mesalazine, immunosuppressants such as azathioprine or methotrexate and corticosteroids) and biologics [anti-tumor necrosis factor (anti-TNF), anti-integrins, and anti-interleukins (IL)], with small molecules (Janus kinase inhibitors and sphingosine 1-phosphate receptor modulators) having recently been added to the possible advanced treatments[2-5].

After the onset of therapy, the treatment of patients with IBD is focused on achieving therapeutic goals, which include improvement or normalization of clinical, biochemical, endoscopic variables, and also the quality of life and disability[6]. Despite the multiple treatments available, about 40% of patients are refractory to several treatments with different mechanisms of action, and these patients present with persistent symptoms that often have a great impact on their quality of life, due to the need for hospitalization and the requirement of surgery, which has to be carried out several times in some cases[7].

Extraintestinal manifestations (EIM) are present in about one-third of patients after diagnosis[8]. These mainly involve osteoarticular and dermatological manifestations. Some EIM are independent of IBD activity and require independent therapeutic management. In addition, some patients have multiple comorbidities throughout the course of the disease associated with prolonged corticosteroid treatment (diabetes, osteoporosis, adrenal insufficiency, and others), which is frequently used in these patients with a suboptimal response to advanced treatments[9].

Different studies have described a therapeutic window of opportunity, which implies the early use of advanced treatment in patients with IBD, especially in patients with early CD (< 2 years)[10]. These interventions are associated with a decrease in the progression of intestinal damage and complications such as stenosis and fistulas, and consequently reducing the need for hospitalization and surgery[10]. Finally, patients with long-standing IBD with persistent inflammatory activity represent a group at higher risk for the development of colorectal cancer, which develops by a different sequence to that of non-IBD colorectal cancer[11]. It has also been described that a better control of inflammatory activity may have an impact the development of this complication during long-term evolution[12,13].

DEFINITION AND INDICATIONS OF DUAL THERAPY

The development of new molecules and the implementation of new strategies are necessary to achieve better control of IBD activity in patients who are refractory to currently available treatments[14]. However, there are multiple pathways of inflammatory activity activated in patients with IBD, and for this reason, treatment with monotherapies may not be sufficient for the management of all patients[15]. Related to this, there are many scenarios in medicine in which dual therapy is used in both the induction and maintenance of treatment. This strategy involves the combination of two or more treatments with the aim of achieving optimal control of pathologies with different therapeutic targets. Indeed, this modality has seen great development in oncological or hematological treatments[14]. Similarly, in patients with rheumatologic pathologies, this approach is used in some patient subgroups [16]. This approach is also applicable to patients with refractory IBD to advanced treatments (dual therapy) by using two biologics simultaneously or a biologic in combination with a small molecule[16,17]. In patients with IBD, there are two distinct scenarios in which it can be used: (1) Patients with

refractory IBD without EIM; and (2) patients with IBD in remission, but with active EIM or immune-mediated inflammatory diseases (IMID)[18].

EVIDENCE RELATED TO DUAL THERAPY

The first clinical trial that assessed a combination of biologics was developed in 2007[19,20]. Later, in 2010, the SONIC trial demonstrated that the association of infliximab and azathioprine is more effective compared with either infliximab or azathioprine monotherapy in CD patients, since which time multiple publications have described the results of different combinations of advanced drugs in patients with UC and CD, both in adults and in the pediatric population[21-35] (Figure 1). These combinations have varied according to the availability and practical experience of the drugs that were approved after the anti-TNFs. Table 1 shows the data from publications related to drug combination in patients with IBD. A major limitation of the dual therapy data is that they are mostly retrospective[19]. For this reason, the definitions of response evaluation (clinical, endoscopic and biochemical) are abbreviated and with the exception of few series are only described for short periods[33]. In addition, the definition of complications and the requirement for hospitalization and surgery can be subject to biases related to the follow-up time and the clinical condition prior to the start of the combined treatment[16]. Also, the differential evaluation of this strategy in patients with UC *vs* CD is not reported in many publications, which makes assessment difficult in some cases. Finally, some series include data on patients who received more than one combination, and it is possible that the effectiveness and adverse events could be different depending on the sequencing order of these combinations.

Effectiveness

The partial or complete response in patients with indication for dual therapy for refractory IBD has been evaluated using different meta-analyses[16,19,36]. In these studies, the patients included were mainly those with CD (70%), and in the great majority, the indication for dual therapy was for refractory endoluminal activity[16]. Overall, the observed clinical response varied between 60% and 84% in most of the publications[16,19]. However, clinical remission, which is a difficult clinical situation to achieve considering that these are multi-refractory patients, ranged between 47% and 80% of the patients who received combined therapy[16,18]. The therapeutic response of the different combinations has not been reported to reveal significant variation with respect to the main indication (refractory luminal activity *vs* active EIM or IMID)[16]. Persistence in the treatment of dual therapy varies according to the follow-up period. It has been published that globally 45% of patients may discontinue the dual scheme during its evolution, with loss of response being the main cause (64%) and intolerance together with adverse effects representing a smaller percentage (12%)[33]. It is noteworthy that in a recent study, 21% of patients were able to discontinue one of the drugs in the combination without impacting the subsequent evolution[33]. It is important to mention that many series have included a recycling strategy. This involves the use in the combination of a drug, which the patient did not respond to[14]. Several publications have mentioned such a situation, and have observed that the response in these patients was similar to that observed in those who had not been previously exposed to that drug[18]. This strategy requires further evolution, especially in areas with limited resources for access to new advanced treatments.

Safety

The combination of two biologics or a biologic plus a small molecule has been associated with a higher rate of complications in other indications[17,18]. This has been observed in studies of patients with rheumatologic diseases who received combination therapy[14]. However, in these series, a significant percentage of patients received different treatments with medications that present a higher rate of adverse events, such as the use of rituximab, abatacept, and tocilizumab[18]. On the other hand, in patients with IBD, most of the proposed combinations include drugs with a high relevant safety profile such as vedolizumab or ustekinumab, which are used in both the pediatric and adult populations[25,29]. In a recent meta-analysis, the presence of adverse events varied from 6%-24% according to the combinations[16]. However, the presence of severe adverse events with indication for hospitalization or surgery was only present in 0%-12% of patients[16]. Within these severe adverse events, 75% were due to both intestinal and soft tissue infections[16]. In a recently published European series, a higher number of infections requiring hospitalization was observed in patients who received anti-TNF, corticosteroids, and immunomodulators, and who had a concomitant diagnosis of IMID/EIM (most frequently ankylosing spondylitis)[33]. Nevertheless, in this series, these complications developed only in patients with CD. Importantly, no case of reactivation of herpes zoster has been reported in any publication. Although one case of herpetic meningoencephalitis was diagnosed in a 43-year-old patient with CD who had received a combination including certolizumab, vedolizumab, and methotrexate, this was resolved after treatment[33]. Finally, one incident case of benign skin neoplasia (clear cell acanthoma) and one case of recurrence of basal cell skin cancer were reported[33,35]. No other cancers or treatment-related deaths have been reported.

Table 1 Publications including 2 or more adult or pediatric inflammatory bowel disease patients with use of combination therapy

Ref.	Study type (type of patients)	Disease (number of patients)	Combinations	Efficacy	Adverse events
Sands *et al* [20], 2007	Randomized controlled trial (adults)	CD (52)	NAT + IFX	Remission 37%	Headache, CD exacerbation, nausea, nasopharyngitis
Buer *et al* [22], 2018	Prospective cohort (adults)	CD (4), UC (6)	9 IFX + VDZ, 1 ADA + UST	Remission 100 %	3 upper airway infections
Mao *et al* [23], 2018	Case series (adults)	CD (4)	1 TNFi + UST/VDZ, 1 VDZ + UST, 2 VDZ + GOL	Remission 3/4	1 hand, foot and mouth disease, 1 influenza, 1 *Clostridiodes difficile*
Kwapisz *et al*[24], 2020	Retrospective cohort (adults)	CD (14), UC (1)	8 VDZ + TNFi, 5 VDZ + UST, 2 UST + TNFi	Improvement 11/15	*Salmonella, Clostridiodes difficile*, 4 infections, arthralgia
Olbjørn *et al* [25], 2020	Retrospective cohort (pediatrics)	CD (9), UC (4)	8 IFX + VDZ, 5 IFX + UST	Remission 9/13	Elevated transaminases, eczema, skin infection
Fumery *et al* [26], 2020	Case series (adults)	CD (5), UC (2)	5 TNFi + UST, 2 TNF + VDZ	Remission 6/7	No
Glassner *et al*[27], 2020	Retrospective cohort (adults)	CD (30), UC (18), IBD-U (1)	7 VDZ + TNFi, 25 VDZ + UST, 9 TOF + TNFi, 8 TOF + VDZ, 3 TOF + UST	Remission 50%	3 bacterial enteric infections (*E. coli*), 3 *Clostridiodes difficile*, 1 peristomal cellulitis, 2 abdominal wall abscesses
Privitera *et al*[28], 2020	Retrospective cohort (adults)	CD (11), UC (5)	3 VDZ + UST, 9 VDZ + TNFi/other, 3 VDZ + UST	Clinical response 43%	1 cutaneous reaction, 1 drug-induced liver injury, 1 perianal abscess
Yang *et al* [29], 2020	Retrospective cohort (adults)	CD (22)	8 VDZ + UST, 13 VDZ + TNFi, 3 UST + TNFi	Remission 41%	1 drug induced lupus, 1 pneumonia, 1 *Clostridiodes difficile*, 1 acinetobacter bacteremia
Alayo *et al* [30], 2021	Retrospective cohort (adults)	CD (10), UC (25)	24 VDZ + TOF, 5 TOF + UST	Remission 70% at 26 wk	1 *Clostridiodes difficile*, 1 candida esophagitis, 1 abnormal lipid profile
Dolinger *et al*[31], 2021	Retrospective cohort (pediatrics)	CD (7), UC (8), IBD-U (1)	9 VDZ + TOF, 4 VDZ + UST, 3 UST + TOF	Remission 12/16	1 septic arthritis, 1 deep vein thrombosis
Llano *et al* [32], 2021	Retrospective cohort (adults)	CD (3), UC (10), IBD-U (1)	3 UST + VDZ, 2 VDZ + TNFi, 9 VDZ + TOF	Clinical or biochemical remission 50%	2 *Clostridiodes difficile*, 2 pneumonia, 3 abnormal lipid profile
Goessens *et al*[33], 2021	Retrospective cohort (adults)	CD (58), UC (40)	41 VDZ + TNFi, 21 VDZ + UST, 11 UST + TNFi, 1 TOF + TNFi, 13 TOF + VDZ, 17 other	Clinical response 44%	10 serious or opportunistic infections
Howard *et al* [34], 2022	Case series (pediatrics)	CD (3)	3 VDZ + UST	Clinical remission 100%	Not reported
Lee *et al*[35], 2022	Retrospective cohort (adults)	CD (19)	7 TOF + VDZ, 11 TOF + UST, 1 TOF + TNFi	Remission 60%	1 basal cell carcinoma

ADA: Adalimumab; CD: Crohn's disease; GOL: Golimumab; IBD-U: Inflammatory bowel disease-unclassified; IFX: Infliximab; NAT: Natalizumab; TNFi: Tumor necrosis factor inhibitor; TOF: Tofacitinib; UC: Ulcerative colitis; UST: Ustekinumab; VDZ: Vedolizumab.

Data in pediatric population

Different case series in pediatric patients have reported results with various combinations in both CD and UC[37]. In one study, 75% of patients with luminal activity achieved a clinical remission free of corticosteroids at 6 mo, with the median time to achieve this goal being 88 d[31]. Interestingly, another potential indication that has been described in pediatric patients is the use of dual therapy (vedolizumab and tofacitinib) in patients with acute severe UC[31]. Nevertheless, more data are needed to explore this indication in an urgent and severe situation in patients with IBD. Different adverse events have been described in pediatric patients, but in general there are less frequent than in adult patients[25]. In a series of 16 pediatric patients, 1 (6%) patient presented septic arthritis and subsequent deep vein thrombosis[31].

NEW HORIZONS

It is necessary to establish new strategies for the use of advanced treatments in patients with refractory IBD, which must take into account health costs in order to be sustainable[14]. The sequencing of

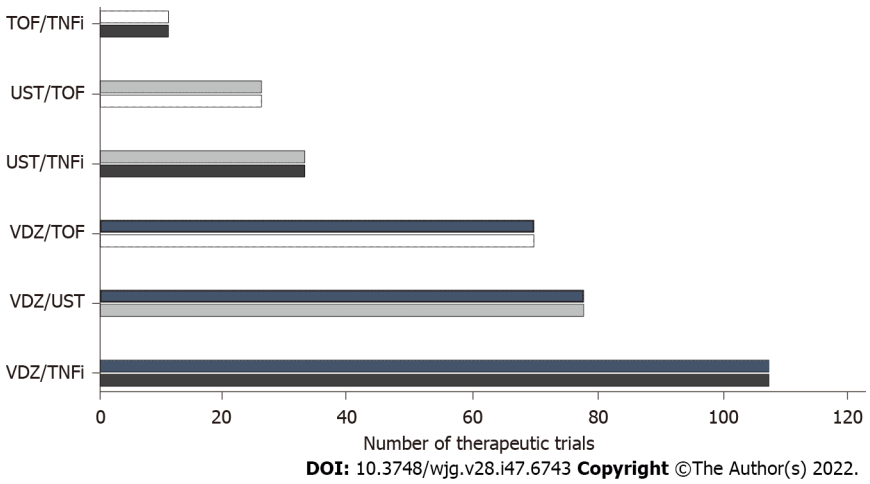

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Figure 1 Number of therapeutic trials described in studies including 2 or more inflammatory bowel disease patients. TNFi: Tumor necrosis factor inhibitor; TOF: Tofacitinib; UST: Ustekinumab; VDZ: Vedolizumab.

biologics or small molecules in patients in remission is a strategy that probably results in a better cost balance. Related to this, some series described patients who achieved remission with two biologics, with the subsequent suspension of one of these (usually anti-TNF) not leading to the presence of disease reactivation during follow-up[22]. In addition, other studies have shown that patients in remission on infliximab were able to maintain their clinical status after initiation of vedolizumab and discontinuation of anti-TNF[38]. The implementation of these strategies requires further research, and in particular, clinical trials are needed to establish their effectiveness during long-term follow-up.

The use of artificial intelligence and the implementation of new biomarkers in the future will possibly be able to differentiate the patients who will benefit from certain combination schemes. Artificial intelligence may also enable remote monitoring to provide new data as well as algorithms to ensure better decision making in refractory patients[39]. In addition, biomarkers might improve patient stratification. Recent data have shown that HLA-DQA1*05 is non-uniformly distributed in patients with or without anti-TNF failure[40]. Likewise, IL-23 receptor expansion is a mechanism of anti-TNF resistance and is reflected as a secondary loss of response[41]. According to this, the use of ustekinumab may allow to regained response in patients with prior anti-TNF.

It is possible that in the near future new combinations with different effectiveness and safety profile will be described, with the use of ozanimod, upadacitinib, risankizumab, guselkumab, and mirikizumab, among others, expanding the current options[18]. In this regard, it is important to note that future clinical trials will be developed to compare current therapy with the combination of two biologic treatments (golimumab and guselkumab) or the combination of two biologics (vedolizumab and adalimumab) and an immunomodulator (methotrexate)[42-44]. Moreover, the design of the new pivotal studies has been modified. Recently, a phase 2 study in patients with CD compared different doses of guselkumab with placebo but also included a ustekinumab arm as this provides better comparative information[45].

Another point to consider is that some good results have been reported after the change of formulation (from intravenous to subcutaneous) of the same drug such is the case of as infliximab or vedolizumab[46,47]. This could be important in future combinations, since it would facilitate logistics and reduce associated costs. In addition to the combination of biological drugs or small molecules, the future role of other approaches should be determined, such as the use of probiotics and gut flora regulators as well as the role of microbiota transplantation[48,49].

Finally, the development of more real-life evidence will be of great importance. Currently most of the data comes from Europe and North America[16]. In this sense, it would be very useful to develop international registries involving several countries currently experiencing a clear increase in the incidence of IBD, such as Latin America and Asia, and which have greater difficulty in accessing advanced treatments[50-52]. In this regard, the costs associated with dual therapy are the main limitation to access, which restrict the provision of a personalized treatment in patients with indication for this strategy[53]. Moreover, it is of great relevance to inform the health insurance of these patients about the objectives and advantages of the dual therapy strategy to obtain the appropriate approval in a timely manner for the indication.

CONCLUSION

The combination of biologics and/or small molecules is a strategy applicable to refractory IBD patients in two distinct scenarios: (1) Refractory active luminal disease without extraintestinal manifestations; and (2) patients with IBD in remission, but with active extraintestinal manifestations or immune-mediated inflammatory diseases. The observed clinical response using this strategy varied between 60% and 84% in most of the publications, and severe adverse events were observed in a few patients. However, most of the data on dual therapy are retrospective and with short-term follow-up. New clinical trials are needed to establish dual therapy effectiveness and safety during long-term follow-up. Finally, it is expected that new combinations using new drugs with different efficacy and safety profiles will be described in the coming years, expanding the current options.

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ORIGINAL ARTICLE

Basic Study

Interleukin-34 deficiency aggravates development of colitis and colitis-associated cancer in mice

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Abstract

BACKGROUND

Although expression of interleukin (IL)-34 is upregulated in active ulcerative colitis (UC), the molecular function and underlying mechanism are largely unclear.

AIM

To investigate the function of IL-34 in acute colitis, in a wound healing model and

in colitis-associated cancer in IL-34-deficient mice.

METHODS

Colitis was induced by administration of dextran sodium sulfate (DSS), and carcinogenesis was induced by azoxymethane (AOM). Whether the impact of IL-34 on colitis was dependent on macrophages was validated by depletion of macrophages in a murine model. The association between IL-34 expression and epithelial proliferation was studied in patients with active UC.

RESULTS

IL-34 deficiency aggravated murine colitis in acute colitis and in wound healing phase. The effect of IL-34 on experimental colitis was not dependent on macrophage differentiation and polarization. IL-34-deficient mice developed more tumors than wild-type mice following administration of AOM and DSS. No significant difference was shown in degree of cellular differentiation in tumors between wild-type and IL-34-deficient mice. IL-34 was dramatically increased in the active UC patients as previously reported. More importantly, expression of IL-34 was positively correlated with epithelial cell proliferation in patients with UC.

CONCLUSION

IL-34 deficiency exacerbates colonic inflammation and accelerates colitis-associated carcinogenesis in mice. It might be served as a potential therapeutic target in UC.

Key Words: Interleukin-34; Ulcerative colitis; Mucosal healing; Colitis-associated cancer; Macrophage; Murine model

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Core Tip: This study highlights the role of interleukin (IL)-34 in acute experimental colitis and wound healing phase in mice. We found that IL-34 did not drive inflammatory response and tissue destruction in physiological conditions, but protects the host from inflammatory injury and reduces the risk of colitis-associated cancer. IL-34 might serve as a potential therapeutic target for inducing mucosal healing in treatment of ulcerative colitis (UC) and reducing colitis-associated cancer in UC.

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INTRODUCTION

Ulcerative colitis (UC) is a chronic progressive recurrent intestinal inflammatory disorder characterized by bloody diarrhea and abdominal pain[1]. UC is an important promoter of colorectal cancer[2]. The exact pathogenesis of UC remains unclear. It is currently assumed that it involves immunological derangement of the gut microbiota in genetically predisposed individuals after exposure to environmental factors[1]. Cytokines exert multiple effects and participate in the pathogenesis of colitis[3]. Identification of distinctive cytokines involved in immunopathogenesis of UC has become a hot spot for the development of biological therapies[4].

Colony-stimulating factor-1 receptor (CSF-1R) signaling regulates intestinal and colon development, gut homeostasis and inflammatory reaction[5]. Interleukin (IL)-34 was discovered in 2008 as a specific ligand for CSF-1R independent of CSF-1[6,7]. Human IL-34 is a homodimeric glycoprotein that consists of 242 amino acids produced by various cell types including epithelial, endothelial and immune cells, and fibroblasts[8]. Secreted IL-34 binds to the extracellular domains of CSF-1R and protein-tyrosine phosphatase, the other receptor for IL-34, which is regulated by syndecan-1, which results in autophosphorylation of its intracellular tyrosine residues and activates several signaling pathways controlling cell biological function[8,9]. IL-34 functions as a pivotal regulator in cell differentiation, proliferation and survival in the mononuclear phagocyte system[10]. Additionally, IL-34 mediates the crosstalk between the innate and adaptive immune systems during inflammation[8,11].

Under normal circumstances, IL-34 is primarily expressed in a tissue-specific manner in keratinocytes and neurons[8]. In pathological status, the expression pattern is changed. Increased IL-34 expression positively associates with disease progression, severity and chronicity in autoimmune diseases such as

rheumatoid arthritis and Sjogren's syndrome[12]. In contrast, IL-34 has demonstrated beneficial effects in other diseases. IL-34 has been identified as a new mediator to induce transplant tolerance by targeting suppressive T regulatory cells[13]. Therefore, the multiple function of IL-34 in different diseases is complex, disputable, and context-dependent[8].

The involvement of IL-34 in inflammatory bowel disease (IBD) has also drawn attention recently. Higher expression of IL-34 in human ileum compared with colon has been revealed[14]. In human and experimental colitis, the mRNA and protein level of IL-34 is markedly increased in inflamed mucosa compared to that in the matched normal mucosa and in healthy controls[14]. Recently, it has been proposed that IL-34 plays a prominent role in intestinal fibrogenesis *via* a p38 mitogen-activated-protein-kinase-dependent mechanism in Crohn's disease[15]. *In vitro,* tumor necrosis factor (TNF)-α significantly upregulates expression of IL-34 in lamina propria mononuclear cells (LPMCs) *via* NF-kB signaling. Intriguingly, LPMCs treated with IL-34 increase expression of proinflammatory cytokines such as IL-6, TNF-α and chemokine CC ligand 20[14]. Accordingly, IL-34 neutralization decreases synthesis of TNF-α and IL-6 in IBD mucosal explants[14]. Therefore, it has been noted that IL-34 sustains gut inflammation *via* regulating positive feedback of proinflammatory cytokine production *in vitro*[8, 14]. On the contrary, IL-34 has previously demonstrated immunosuppressive characteristics that contribute to inflammation improvement by inducing differentiation of monocytes into M2 phenotype [16]. M2 macrophages mediate immunotolerance and promote wound healing in experimental colitis *in vivo*[17,18]. IL-34 is upregulated in colon cancer and sustains the protumorigenic signals by inducing proliferation of cancer cells *via* extracellular signal-regulated kinase (ERK) 1/2 or cancer-associated fibroblasts[19,20]. Whether IL-34 is "friend or foe" in the pathogenesis of UC remains to be explored. In this study, we investigated the potential role of IL-34 in experimental colitis, colitis-associated carcinogenesis and UC.

MATERIALS AND METHODS

Animal experiment

IL-34-deficient mice were generated in C57BL/6J mice by using CRISPR/Cas9 technology (Beijing Cas Gene Biotech, Beijing, China). Gene targeting technology was applied to delete exons 3-5 in the *IL-34* gene and led to frameshift mutation of *IL-34* gene. IL-34-deficient and C57BL/6J wild-type mice were bred and maintained in a specific-pathogen-free animal facility in the Laboratory Animal Center of Nantong University. All animal experiments were approved by the Institutional Ethics Committee of Nantong University (Date: 21/12/2015, Number: S20151221-908).

Acute colitis was induced by oral administration of 3% (M/V) dextran sulfate sodium (DSS, MW: 36 000-50 000; MP Biologicals, LLC, California, United States) in drinking water for 7 d. The mice were killed to obtain colon tissues at the indicated time or until day 16 to record the mortality. The murine wound healing model was established by oral administration of 3% DSS for 5 d and then switched to normal water for the following 5 d. The mice were killed on day 8 or 10. Colitis-associated cancer was induced by administration of the carcinogen azoxymethane (AOM; Sigma, Darmstadt, Germany) and repeated administration of DSS. The mice were given a single injection of AOM (10 mg/kg). Seven days later, mice were administrated 1.25% DSS (w/v) in drinking water for seven consecutive days, and fresh drinking water for 14 d. Seven days of DSS and 14 d of fresh water was repeated four times as a cycle [21]. The mice were killed, and the incidence rate of tumors was analyzed. Macrophage depletion from murine colons *in vivo* was performed as described previously[22]. In brief, 200 μL clodronate liposomes (Liposoma Research, Amsterdam, Netherlands) were intraperitoneally injected into mice 2 d prior to onset of experimental colitis and every 2 d during the process.

The percentage of body weight change of each mouse was recorded throughout the duration of DSS administration. Fresh feces from mice were collected for occult blood tests using a fecal occult blood kit (Nanjing Jiancheng Bioengineering, Jiangsu, China). The clinical score index of the murine model consisted of stool consistency and fecal occult blood, as previously described[23].

Human tissue

Human tissue specimens were obtained from 40 adult patients with active UC and 20 healthy controls during colonoscopy in Nantong Third People's Hospital Affiliated to Nantong University and Affiliated Hospital of Nantong University between January 2014 and August 2015. Histopathology was evaluated by an experienced pathologist. This study was approved by the Ethics Committee of Nantong Third People's Hospital Affiliated to Nantong University and Affiliated Hospital of Nantong University, and all people signed an informed consent form. All patients met the diagnostic criteria that were in line with the Consensus on Diagnosis and Management of Inflammatory Bowel Disease (2018, Beijing, China). The patients did not receive any therapy before colonoscopy. More details on the patients' characteristics can be found in Supplementary Table 1.

Histopathology

The entire colon was harvested for measuring the length. The colon was washed in phosphate-buffer saline and fixed in 10% formaldehyde solution for 24 h. Hematoxylin-eosin (H&E) staining was performed on the tissue sections. The inflammatory score was as follows: Presence of occasional inflammatory cells in the lamina propria was scored as 0; increased numbers of inflammatory cells in the lamina propria was scored as 1; confluence of inflammatory cells extending into the submucosa was scored as 2; and transmural extension of the infiltrate was scored as 3. For tissue damage score, no mucosal damage was scored as 0; lymphoepithelial lesions were scored as 1; surface mucosal erosion or focal ulceration was scored as 2; and extensive mucosal damage and extension into deeper structures of the bowel wall was scored as 3. The combined histological score was calculated by inflammatory and tissue damage score, and ranged from 0 (no changes) to 6 (extensive infiltration and tissue damage).

Immunohistochemistry

The colonic tissues were sectioned into 4-μm slices following fixation with 10% formaldehyde and embedded in paraffin. Immunohistochemical staining was conducted as in our previous study[24]. Tissue slices were incubated with 3% H_2O_2 for 15 min after deparaffinization. Antigen was retrieved in citrate buffer (pH 6.0; Maxim Biotechnologies, Fuzhou, China) in a microwave for 10 min. Nonimmune 10% goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA, United States) was used to block nonspecific reactions. The sections were incubated with primary antibodies: rabbit anti human/mouse IL34, 1:100 (Abcam, Cambridge, MA, United States); rabbit anti human/mouse Ki-67 antibody, 1:200 (Abcam); rabbit anti mouse CSF1-R 1:1000 (Abcam); and rabbit anti-mouse CD68, 1:100 (Boster Biological Technology, Wuhan, China) overnight at 4 °C. On the second day, the sections were incubated with horseradish-peroxidase-conjugated secondary antibody (Shanghai Long Island Biotechnology, Shanghai, China) for 30 min at room temperature. 3, 3′-diaminobenzidine (Maxim Biotechnologies) was applied for 30 s. Hematoxylin was applied for contrast staining. Proliferation index of Ki-67 was defined as the percentage of Ki-67-positive cells in crypts (CK4) within the random visual scope. Positive index of CSF1-R was defined as the percentage of CSF1-R-positive cells in colonic mucosa within the random visual scope. All the tissue sections were analyzed by optical microscopy (IX73; Olympus, Tokyo, Japan) by experienced pathologists.

TUNEL assay

Colonic tissue sections were prepared as described above. Apoptosis was analyzed by fluorescence microscopy according to standard procedure using *in situ* cell death detection (Roche, Basel, Switzerland). Five randomly optical fields were chosen for further analysis.

RNA isolation and quantitative real-time PCR

Total RNA was extracted from the indicated murine colonic tissues by Trizol Reagent (TaKaRa Bio, Dalian, China). RNA samples were reverse-transcribed into cDNA with a PrimeScript™RT Master Mix kit (TaKaRa Bio). cDNA samples were detected using a SYBR®Premix Ex Taq™II kit (TaKaRa Bio) on CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, United States). PCR was performed at 95 °C for 30 s, and the samples were subjected to 40 cycles of amplification at 95 °C for 5 s and 60 °C for 30 s. Expression of target genes was normalized to β-actin. Gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. All the primers are listed in Supplementary Table 2.

Statistical analysis

Data are presented as mean ± SD. The difference between more than two groups was analyzed using one-way analysis of variance and comparison of two groups was carried out using a *t*-test. Differences in survival between two groups were analyzed by Kaplan-Meier test. All statistical analyses were performed using GraphPad Prism 6.0 (San Diego, CA, United States). $P < 0.05$ was considered statistically significantly.

RESULTS

IL-34 is elevated in colitis and colitis-related cancer

We analyzed IL-34 mRNA expression in gastrointestinal mucosal epithelium in wild-type C57BL/6J mice. Expression of IL-34 was highest in the colon compared with other parts of the digestive tract (Figure 1A). We investigated expression of IL-34 in DSS-induced colitis and AOM-DSS-induced colitis-associated cancer in wild-type mice. Immunohistochemical staining of murine colon tissue revealed that expression of IL-34 was elevated in both acute and chronic colitis, with the highest expression in colitis-associated cancer (Figure 1B and C).

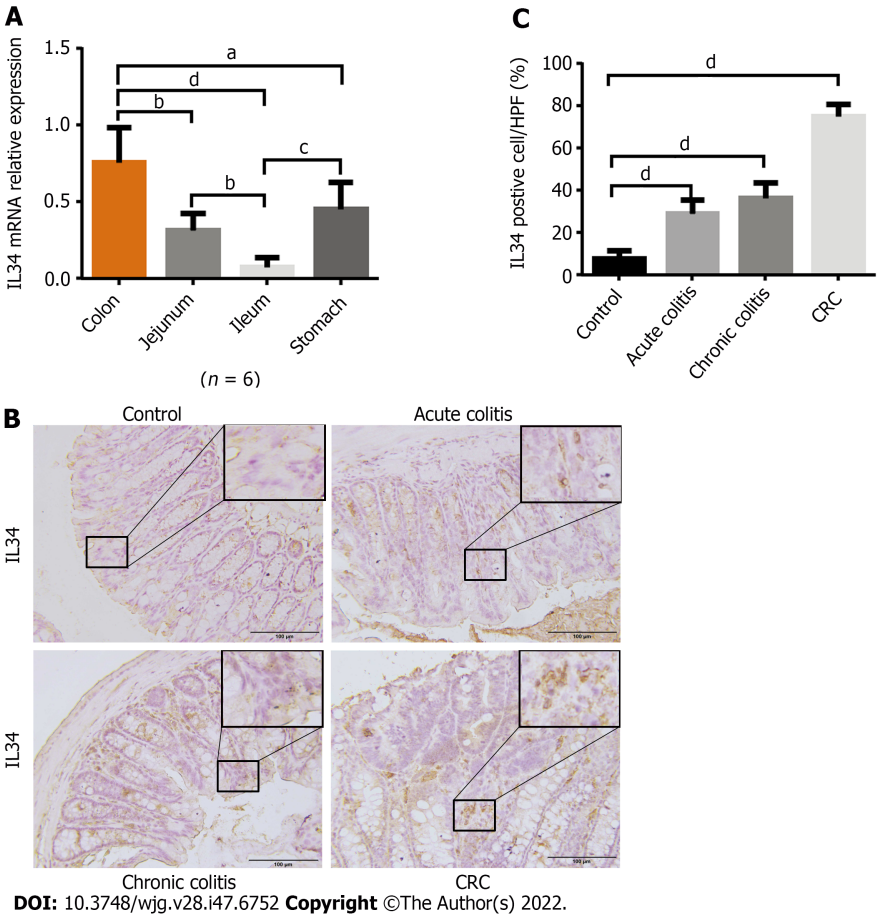

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Figure 1 Interleukin-34 is elevated in colitis and colitis-related cancers. A: Interleukin-34 (IL-34) relative mRNA expression in stomach, jejunum, ileum and colon in wild-type mice (C57BL/6J) (n = 6); B and C: The dynamic of IL-34 expression in dextran sodium sulfate (DSS)-induced colitis and azoxymethane-DSS-induced colorectal cancer in wild-type mice (n = 6 per group); representative IL-34 immunohistochemical staining for healthy control, acute colitis, chronic colitis and colorectal cancer mice (B); the percentage of IL-34-positive cells per high-power field were quantified in colonic tissue of four groups of mice (n = 6 per group) (C). AOM: Azoxymethane; CRC: Colorectal cancer; DSS: Dextran sodium sulfate; IL-34: Interleukin-34. Scale bars = 100 μm. Data depict the mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.005$, $^{d}P < 0.001$.

IL-34 deficiency increases susceptibility to acute DSS-induced colitis

To determine the role of IL-34 in murine acute experimental colitis, IL-34$^{-/-}$ and wild-type mice were fed with 3% DSS in drinking water for 7 d and then killed (Figure 2A). DSS-fed IL-34$^{-/-}$ mice showed significantly greater body weight loss compared to DSS-fed wild-type mice. The average weight loss in DSS-fed IL-34$^{-/-}$ mice was approximately double than that in the wild-type mice (Figure 2B). IL-34$^{-/-}$ mice displayed a significantly higher clinical score compared to wild-type mice (Figure 2C). Strikingly, the mortality of IL-34$^{-/-}$ mice was 100% (10/10), whereas only 20% (2/10) of the wild-type mice died during the experiment, as illustrated by Kaplan-Meier curves (Figure 2D, $P < 0.001$). Following DSS administration, IL-34$^{-/-}$ mice showed remarkably shorter colon compared to wild-type mice (4.83 cm ± 0.13 cm *vs* 6.27 cm ± 0.14 cm, $P < 0.001$), suggesting more severe colitis in IL-34$^{-/-}$ mice (Figure 2E and F). DSS-fed IL-34$^{-/-}$ mice exhibited more severe inflammation with ulceration and necrotic lesions compared to wild-type mice, while there was no difference in the wild-type or IL-34$^{-/-}$ mice fed with normal water. More inflammatory cells infiltrated the lamina propria and submucosa in DSS-fed IL-34$^{-/-}$ mice. More importantly, the normal tissue architecture damage was more severe in DSS-fed IL-34$^{-/-}$ mice compared with wild-type mice (Figure 3A). Semiquantitative score of histopathology confirmed more severe colitis in DSS-fed IL-34$^{-/-}$ mice compared to DSS-fed wild-type mice (9.17 ± 0.31 *vs* 5.60 ± 1.10, $P < 0.001$) (Figure 3B). The expression of CD68, which serves as a characteristic marker for macrophages, was indistinguishable between IL-34$^{-/-}$ and wild-type mice untreated with DSS (Figure 3C and D). CD68 expression was significantly increased in DSS-fed IL-34$^{-/-}$ mice compared to DSS-fed wild-type controls (Figure 3C and D), which indicated worsening colitis. IL-1β, IL-23, and macrophage colony-stimulating factor levels were significantly upregulated in DSS-treated IL-34$^{-/-}$ mice compared to wild-type mice treated with DSS (Figure 3E).

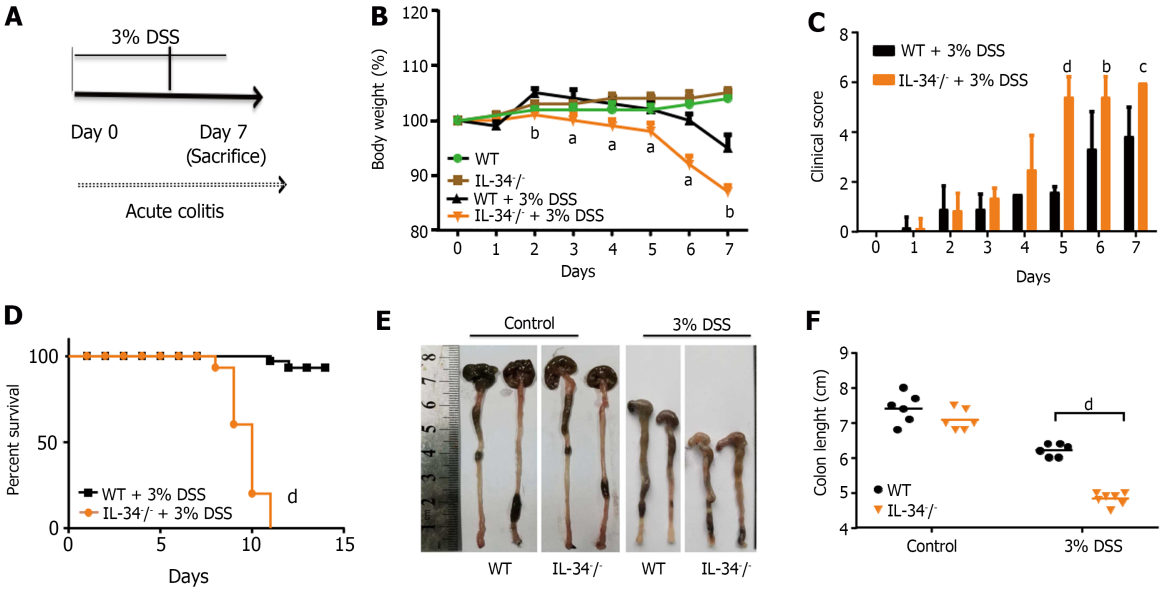

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Figure 2 Interleukin-34 deficiency aggravates acute colitis induced by dextran sodium sulfate. A: Schematic of the design of acute dextran sodium sulfate (DSS)-induced colitis model. IL-34$^{-/-}$ and WT mice were fed with a 3% DSS solution in drinking water for 7 d and then sacrificed (n = 6 or 7 per group); B: Body weight was represented as a percentage of starting weight in IL-34$^{-/-}$ and WT mice after administration of 3% DSS (n = 6 or 7 per group); C: The clinical score of IL-34$^{-/-}$ and WT mice treated with 3% DSS (n = 6 or 7 per group). Data depict the mean ± SD; D: Kaplan-Meier survival curves were plotted in IL-34$^{-/-}$ and WT mice administrated with 3% DSS for 15 d (n = 10 per group); E and F: Colon length was measured in IL-34$^{-/-}$ and WT mice fed with 3% DSS for 7 d (n = 6 or 7 per group). DSS: Dextran sodium sulfate; IL-34: Interleukin-34; WT: Wild-type. Data depict the mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.005$, $^{d}P < 0.001$.

IL-34 deficiency inhibits proliferation and promotes apoptosis of colonic epithelium in DSS-induced colitis

Prior to DSS administration, the levels of colonic epithelium proliferation and apoptosis were both comparable between wild-type and IL-34$^{-/-}$ mice (Figure 4A-D). A marked reduction in colonic epithelial cells stained positive for Ki-67 was detected in DSS-fed IL-34$^{-/-}$ mice compared to DSS-fed wild-type mice (Figure 4A and B). A marked increased in colonic epithelial cell apoptosis was noted in DSS-fed IL-34$^{-/-}$ mice compared to the DSS-fed wild-type mice (Figure 4C and D). We detected expression of three receptors of IL-34 in mouse colonic tissue by real-time quantitative PCR. In DSS-fed IL-34$^{-/-}$ mice, we only observed an increase in the expression of CSF1-R, but the other two receptors of IL34, ptprz1, and syndecan-1, did not change significantly (Figure 4E). By immunohistochemical staining of the mouse colonic tissues, we found that CSF1-R was expressed in epithelial and mesenchymal cells. Compared to the control group, the mice in the DSS-fed group showed an increasing positive index of CSF1-R. Expression of CSF1-R did not show a significant difference between IL-34$^{-/-}$ and wild-type mice untreated with DSS ($P > 0.05$, Figure 4F and G). DSS-fed IL-34$^{-/-}$ mice exhibited a higher index of CSF1-R positivity compared to DSS-fed wild-type mice ($P < 0.01$, Figure 4F and G). CSF1-R acts as a receptor for IL-34 and was compensatorily increased when colitis developed and IL-34 was absent. The results were consistent with the results of quantitative real-time PCR of CSF1R.

Protective effect of IL-34 against acute DSS-induced colitis is not dependent on macrophages

Macrophages were increased in the IL-34$^{-/-}$ mice treated with 3% DSS compared with the wild-type mice. We investigated whether the protective effect of IL-34 in colitis was dependent on macrophages. It is known that IL-34 plays a role in macrophage polarization. We studied the potential role of IL-34 in macrophage polarization by examining the selected markers for M1 and M2 macrophages. Most importantly, there was no difference in the ratio of colonic M2/M1 macrophage markers between DSS-treated wild-type and IL-34$^{-/-}$ mice (Figure 5A). It was speculated that the effect of IL-34 on experimental colitis was not attributed to macrophage polarization. In order to confirm that the protective effect of IL-34 in colitis was not dependent on macrophages, we depleted macrophages by intraperitoneal injection of clodronate liposomes (Clo-lips) in IL-34$^{-/-}$ and wild-type mice (Figure 5B). The macrophage cell marker F4/80 was significantly reduced in colonic mucosa of mice treated with Clo-lips, which confirmed that the liposomes effectively depleted macrophages in the colon (Figure 5C). Despite macrophage depletion, DSS-fed IL-34$^{-/-}$ mice still showed significantly higher clinical score and shorter colon length compared with DSS-fed wild-type mice (Figure 5D-F). Similarly, the histopathological manifestation showed that colitis severity in DSS-fed IL-34$^{-/-}$ mice was more pronounced compared to that in DSS-fed wild-type mice (Figure 5G and H). The inflammatory cytokines remained significantly higher in colonic mucosa of DSS-fed IL-34$^{-/-}$ mice compared with DSS-fed wild-type mice (Figure 5I).

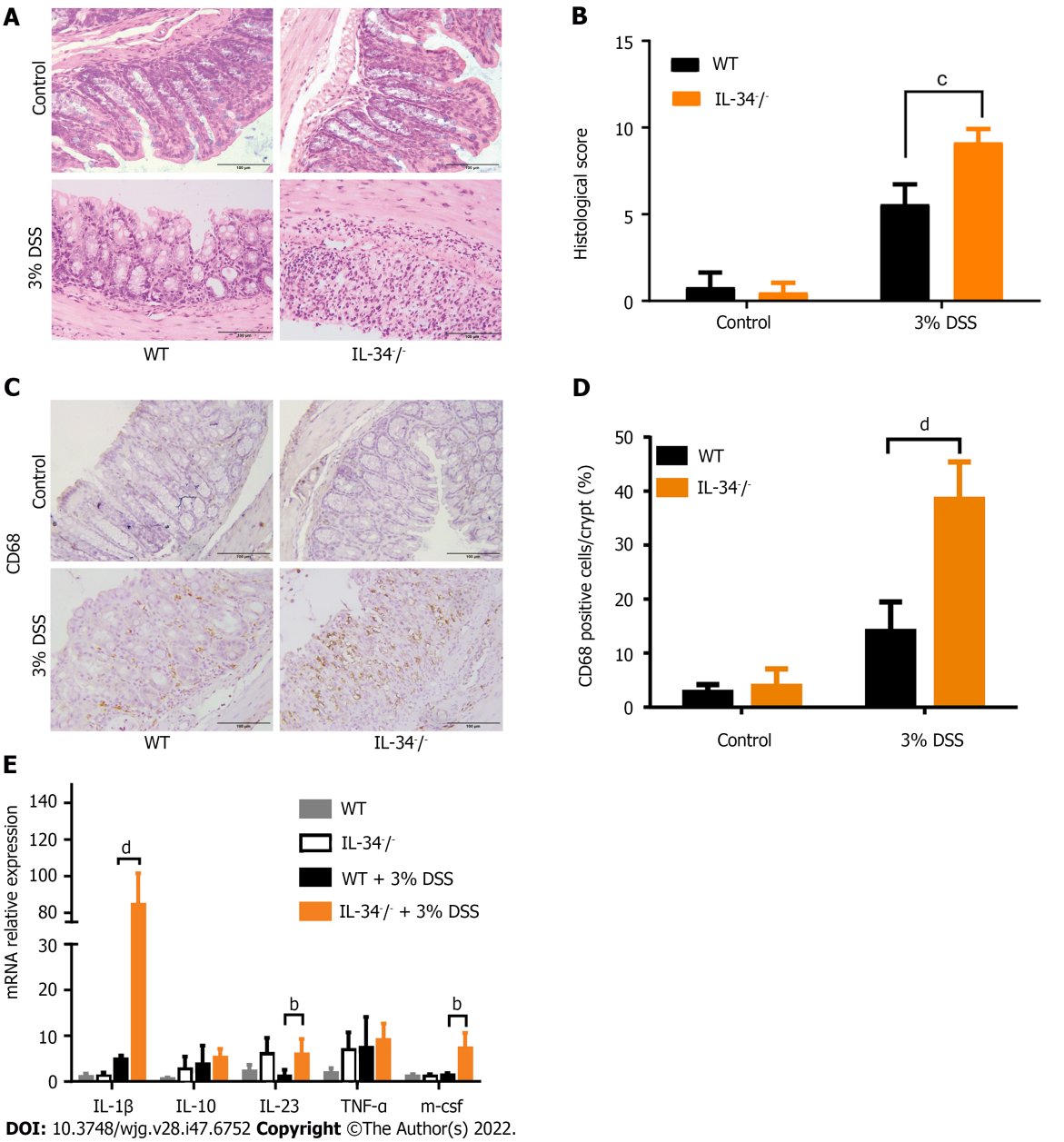

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Figure 3 Interleukin-34 deficiency aggravates acute colitis and increase proinflammatory cytokines. A: Representative microscopic pictures of hematoxylin and eosin-stained colon sections of Interleukin-34 (IL-34)$^{-/-}$ and WT mice fed with 3% DSS for 7 d (n = 6 or 7 per group); B: Histological score for IL-34$^{-/-}$ and WT mice fed with 3% dextran sodium sulfate (DSS); C: Representative photomicrographs of macrophage staining in colon sections of IL-34$^{-/-}$ and WT mice treated with DSS; D: Statistical analysis of CD68-positive cells in IL-34$^{-/-}$ and WT mice treated with DSS; E: mRNA expression of proinflammatory cytokines including IL-1β, IL-10, IL-23, TNF-α, and M-CSF in IL-34$^{-/-}$ and WT mice fed with 3% DSS. DSS: Dextran sodium sulfate; IL: Interleukin; M-CSF: Macrophage colony-stimulating factor; TNF-α: Tumor necrosis factor-α; WT: Wild-type. Scale bars = 100 μm. Data depict the mean ± SD. $^{b}P < 0.01$, $^{c}P < 0.005$, $^{d}P < 0.001$.

IL-34 deficiency delays mucosal healing in a murine model induced by DSS

IL-34$^{-/-}$ and wild-type mice were fed with 3% DSS for 5 d and then switched to normal water for the following 5 d to establish a mucosal healing model. The mice in both groups were killed on day 8 or 10 (Figure 6A). The body weight loss was significantly greater in IL-34$^{-/-}$ mice compared to the wild-type control mice. The body weight increased quickly on day 8 in wild-type mice, while it did not recover until day 10 in IL-34$^{-/-}$ mice (Figure 6B). The clinical score was higher in IL-34$^{-/-}$ mice compared to wild-type mice from day 5. The clinical score decreased rapidly from day 7 in wild-type mice, while it was falling slowly from day 8 in IL-34$^{-/-}$ mice (Figure 6C). IL-34$^{-/-}$ mice showed remarkably shorter colon length compared to wild-type controls on day 8 (5.25 cm ± 0.32 cm *vs* 6.30 cm ± 0.25 cm, $P < 0.01$) and day 10 (5.03 cm ± 0.49 cm *vs* 7.30 cm ± 0.47 cm, $P < 0.005$) (Figure 6D and E). Histopathological analysis showed that IL-34$^{-/-}$ mice exhibited more severe inflammation and tissue damage compared to wild-type mice on days 8 and 10, respectively (Figure 6F). Semiquantitative scoring of histopathology showed that colitis severity in IL-34$^{-/-}$ mice was significantly higher than that in wild-type mice on day 8 (8.13 ± 0.66 *vs* 3.63 ± 0.58, $P < 0.01$) and day 10 (8.45 ± 061 *vs* 2.60 ± 0.58, $P < 0.01$), respectively (Figure 6G). The

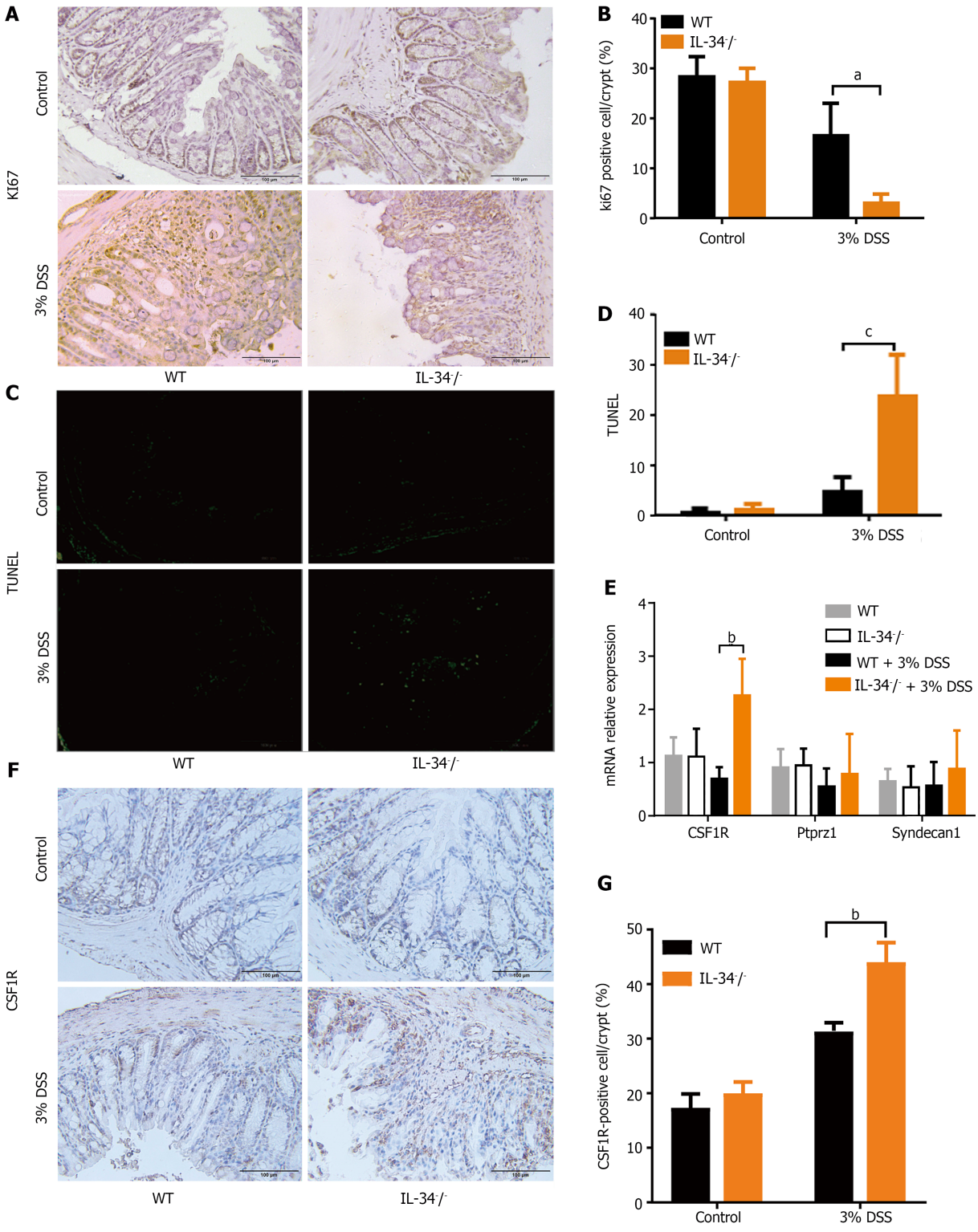

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Figure 4 Interleukin-34 deficiency inhibits proliferation and promotes apoptosis of colonic epithelium in dextran sodium sulfate-induced colitis. A: Representative photomicrographs of Ki-67 immunohistochemical staining for colon sections of IL-34$^{-/-}$ and WT mice treated with dextran sodium sulfate (DSS); B: Percentage of proliferation marker Ki-67 per crypt; C and D: Representative microscopic pictures of TUNEL staining (C) and quantification of TUNEL-positive cells per field (D) in IL-34$^{-/-}$ and WT mice treated with DSS as before; E: mRNA expression of three receptors for IL-34 (CSF1R, ptprz1 and syndecan-1) was detected in IL-34$^{-/-}$ and WT mice treated with DSS (n = 6 or 7 per group); F and G: Representative photomicrographs of CSF1R immunohistochemical staining (F) and percentage of CSF1R-positive cells (G) in colon sections of IL-34$^{-/-}$ and WT mice treated with 3% DSS. CSF1R: Colony-stimulating factor-1 receptor; DSS: Dextran sodium sulfate; IL-34: Interleukin-34; WT: Wild-type. Scale bars = 100 μm. Data depict mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.005$.

number of colonic epithelial cells positive for Ki-67 was markedly decreased in IL-34$^{-/-}$ mice compared to wild-type mice on days 8 and 10 (Figure 6H and I).

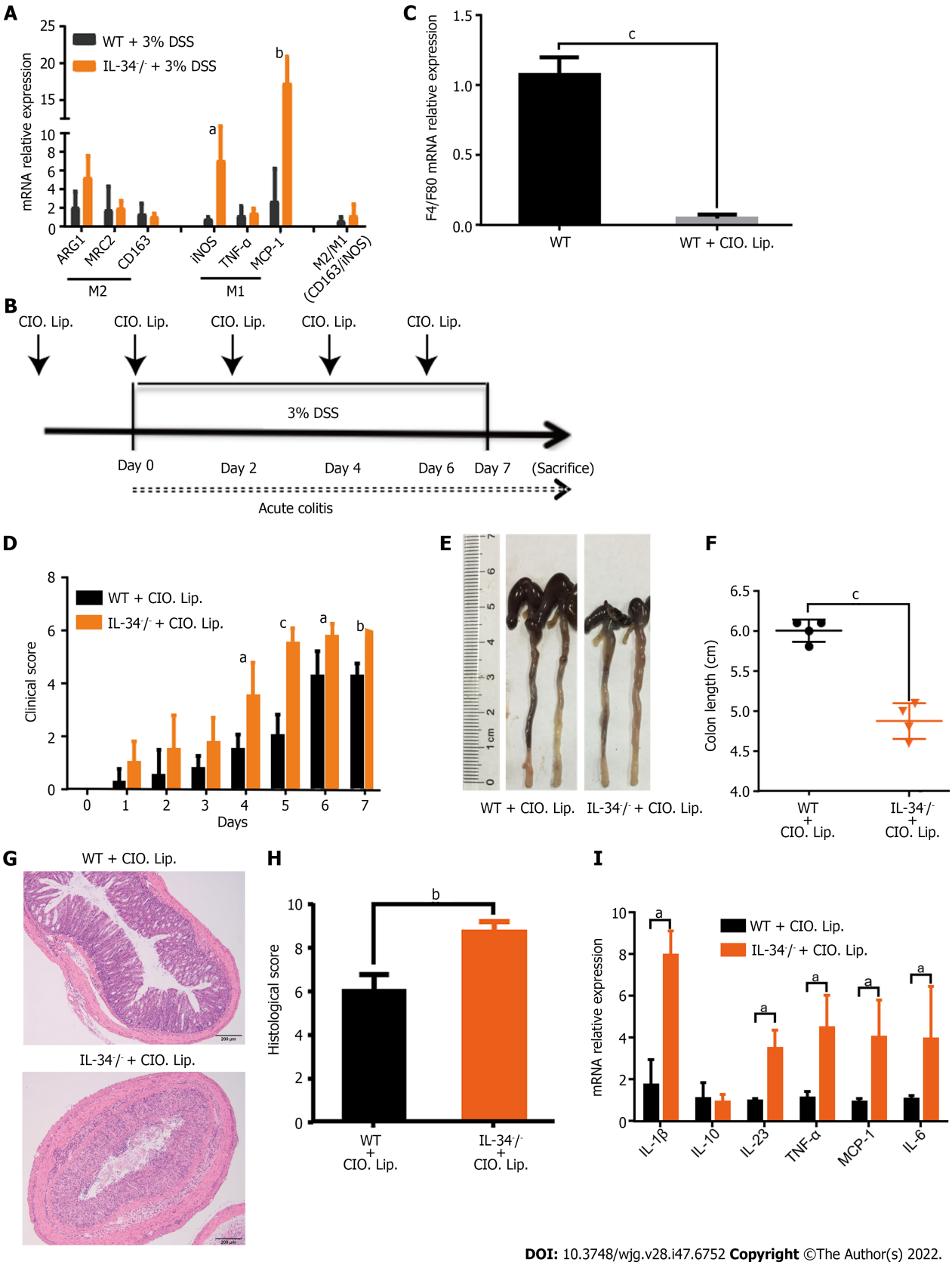

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Figure 5 Protective effect of interleukin-34 against acute dextran-sodium-sulfate-induced colitis is not dependent on macrophages. A: mRNA expression levels of M1 macrophage markers (iNOS, TNF-α and MCP-1) and M2 macrophage markers [ARG1, MRC2 (CD163)] in the colonic mucosa of IL-34$^{-/-}$ and WT mice treated with 3% DSS. M2/M1 ratio (CD163/iNOS) was calculated; B: Experimental design. To deplete macrophages, IL-34$^{-/-}$ and WT mice were treated with Clo-lips 2 d prior to 3% dextran sodium sulfate (DSS) administration and continuing once every 2 d until death (n = 4 per group); C: mRNA expression of macrophage cell marker (F4/80) was detected in colonic mucosa of WT mice treated with Clo-lips (n = 4 per group); D: Clinical score of IL-34$^{-/-}$ and WT mice treated with 3% DSS and Clo-lips (n = 4 per group); E and F: Colon length of IL-34$^{-/-}$ and WT mice treated with 3% DSS and Clo-lips (n = 4 per group); G and H: Representative microscopic pictures (G) and histological scores (H) of IL-34$^{-/-}$ and WT mice fed with 3% DSS and Clo-lips (n = 4 per group); I: mRNA expression of

inflammatory cytokines was detected in IL-34$^{-/-}$ and WT mice treated with 3% DSS and Clo-lips (n = 4 per group). ARG1: Arginase; Clo-lip: Clodronate liposome; CSF1R: Colony-stimulating factor-1 receptor; DSS: Dextran sodium sulfate; IL-34: Interleukin-34; iNOS: Inducible nitric oxide synthase; MCP-1: Monocyte chemoattractant protein-1; MRC2: Mannose receptor C type 2; TNF-α: Tumor necrosis factor-α; WT: Wild-type. Scale bars = 200 μm. Data depict mean ± SD. ^{a}P < 0.05, ^{b}P < 0.01, ^{c}P < 0.005.

IL-34 deficiency promotes AOM/DSS-induced colitis-associated tumorigenesis

To investigate the role of IL-34 in colitis-associated tumorigenesis, wild-type and IL-34$^{-/-}$ mice were treated with AOM and DSS (Figure 7A). No wild-type mice died, whereas a mortality rate of 30% was noted in IL-34$^{-/-}$ mice (Figure 7B). More importantly, a marked difference in tumor number was observed between the two groups. IL-34$^{-/-}$ mice developed a greater number of colon tumors than wild-type mice (Figure 7C and D). Representative H&E staining of colitis-associated cancer in wild-type and IL-34$^{-/-}$ mice is shown in Figure 7E.

IL-34 expression was elevated in inflamed mucosa and was associated with colonic epithelium proliferation in UC patients

We investigated the IL-34 expression pattern and its relationship with colonic epithelium proliferation in active UC patients. There was no age or sex difference between the groups (Supplementary Table 1). Consistent with previous studies, we also observed elevated expression of IL-34 in diseased mucosa of UC patients compared with the normal controls as detected by immunohistochemistry. Importantly, IL-34 was suspected to be predominantly expressed in the colonic stromal tissue (Figure 8A and B). Significantly enhanced colonic expression of proliferation index Ki-67 was detected in UC patients with higher IL-34 expression in colonic mucosa (Figure 8C). There was a positive correlation between IL-34 and Ki-67 expression in UC-inflamed mucosa with a correlation coefficient of 0.60 (P < 0.0001) (Figure 8D). No correlation was found between IL-34-positive and TUNEL-positive cells (data not shown).

DISCUSSION

Mucosal healing has become the goal of therapy because of its association with better prognosis and improved quality of life[25]. However, the underlying molecular mechanism is unclear. Here, we revealed that IL-34 deficiency strongly increased susceptibility to acute DSS-induced colitis and intestinal wounding. Additionally, IL-34 deficiency enhanced AOM/DSS-induced colitis-associated tumorigenesis. Different from the previous reports, our findings suggest the protective role of IL-34 in UC- and colitis-associated cancer and may help to establish a new potential approach for management of UC.

The IL-34 knockout mice used in our study facilitated us to illustrate the role of IL-34 in pathogenesis of UC. Prior to DSS administration, no difference in colon morphological and histological manifestations was observed between IL-34-deficient and wild-type control mice. The results suggest that IL-34 is dispensable for colon homoeostasis under steady state.

In past studies, overexpression of IL-34 stimulated by inflammatory cytokines augmented the production of proinflammatory cytokines *in vitro*, probably raising IL-34 as a pathogenic contributor to sustained colon inflammation[14]. However, the increased expression of IL-34 might be a consequence of inflammation as a protective mechanism rather than a disease cause[16]. Indeed, IL-34 transgenic mice do not display aggravating inflammatory responses in the colon[26]. The precise mechanistic role of IL-34 during colon inflammation has not been clarified yet. Until now, there has been a lack of research on IL-34 involved in UC *in vivo*. A recent study has reported that IL-34 is critical for the suppressive function of CD4^{+} T regulatory cells, and its deficiency leads to increased susceptibility to autoimmunity[27]. We demonstrated for the first time that IL-34 deficiency exacerbates DSS-induced colitis during acute and delayed mucosal healing process and is associated with high colitis-related mortality. We assumed that significantly increased expression of IL-34 in mucosa from patients with active UC might act as an urgent requirement for alleviating inflammation and promoting mucosal healing. Our findings support the protective characteristic of IL-34 during DSS-induced colitis and early healing stage.

Macrophages are critical to mucosal homeostasis in orchestrating innate and adaptive immunity[28]. Depending on the local microenvironment, macrophages differentiated from the peripheral blood monocytes develop towards M1-like or M2-like phenotype macrophages[29]. M1 macrophages secreting proinflammatory cytokines such as TNF-α or IL-12 are considered to amplify the inflammatory response, whereas M2 macrophages producing anti-inflammatory mediators such transforming growth factor-β or IL-10 suppress the inflammatory response[29]. IL-34 has been identified to trigger CSF-1R signaling and induce macrophage differentiation to M2 phenotype *via* activation of the ERK1/2/AKT/AMPK signaling pathway[8], which contributes to mucosal homeostasis maintenance and

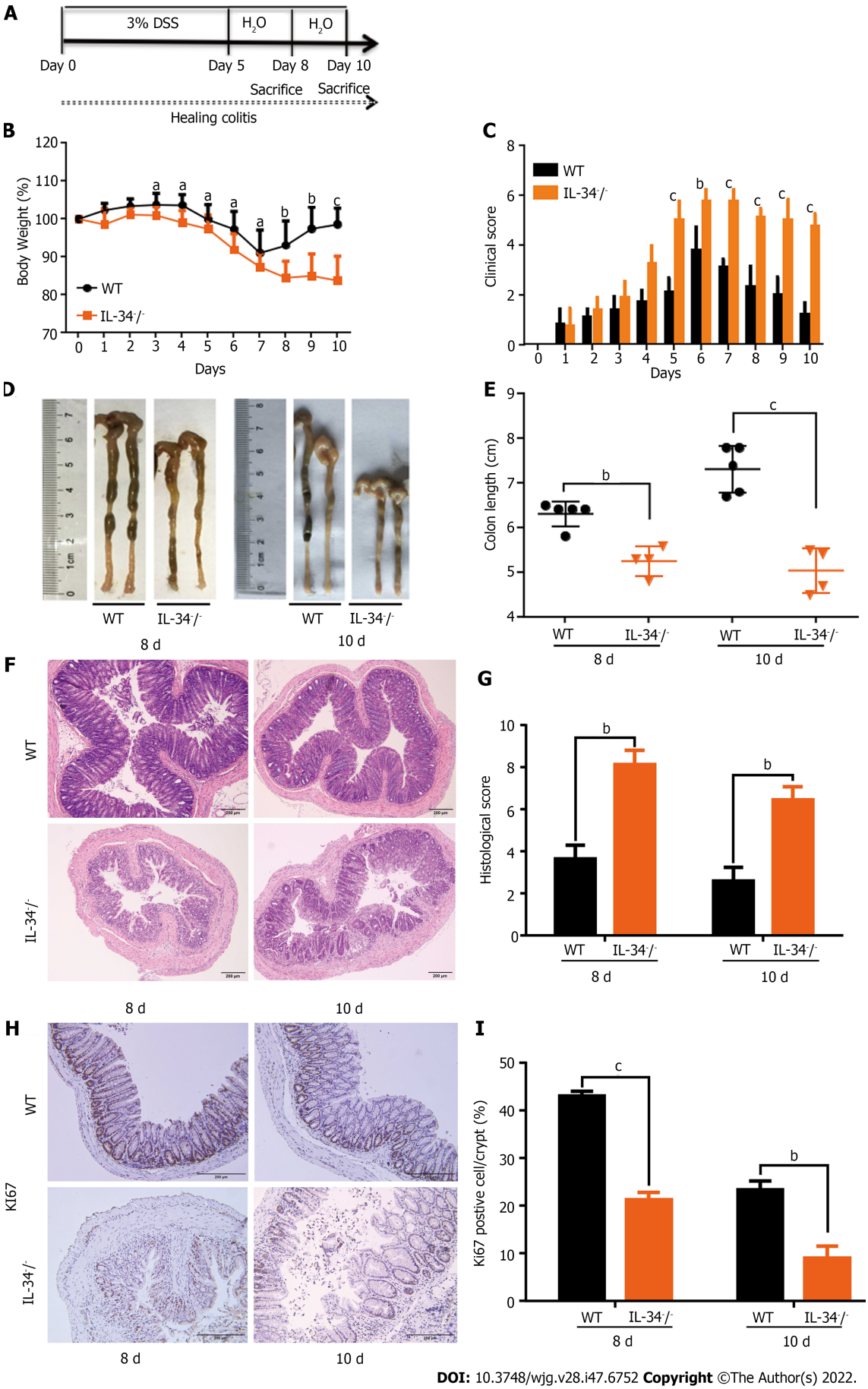

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Figure 6 Interleukin-34 deficiency delays the mucosal healing in murine model induced by dextran sodium sulfate. A: Establishing healing

model chart. The Interleukin-34 (IL-34)$^{-/-}$ and WT mice were fed with 3% dextran sodium sulfate (DSS) for 5 d and switched to normal drinking water for the following 5 d. The mice in both groups were killed on day 8 or 10 (n = 4 or 5 per group); B and C: Body weight (B) and clinical score (C) were determined daily in IL-34$^{-/-}$ and WT mice until day 10 (n = 4 or 5 per group); D and E: The colon length was measured in IL-34$^{-/-}$ and WT mice on day 8 and 10, respectively (n = 4 or 5 per group); F and G: Representative microscopic pictures (F) and semiquantitative histological scores (G) of IL-34$^{-/-}$ and WT mice on day 8 and 10, respectively (n = 4 or 5 per group); H and I: Representative photomicrographs of Ki-67 immunohistochemical staining (H) and percentage of Ki-67-positive per crypt (I) in IL-34$^{-/-}$ and WT mice on day 8 and 10, respectively. DSS: Dextran sodium sulfate; IL-34: Interleukin-34; WT: Wild type. Scale bars = 200 μm. Data depict the mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.005$.

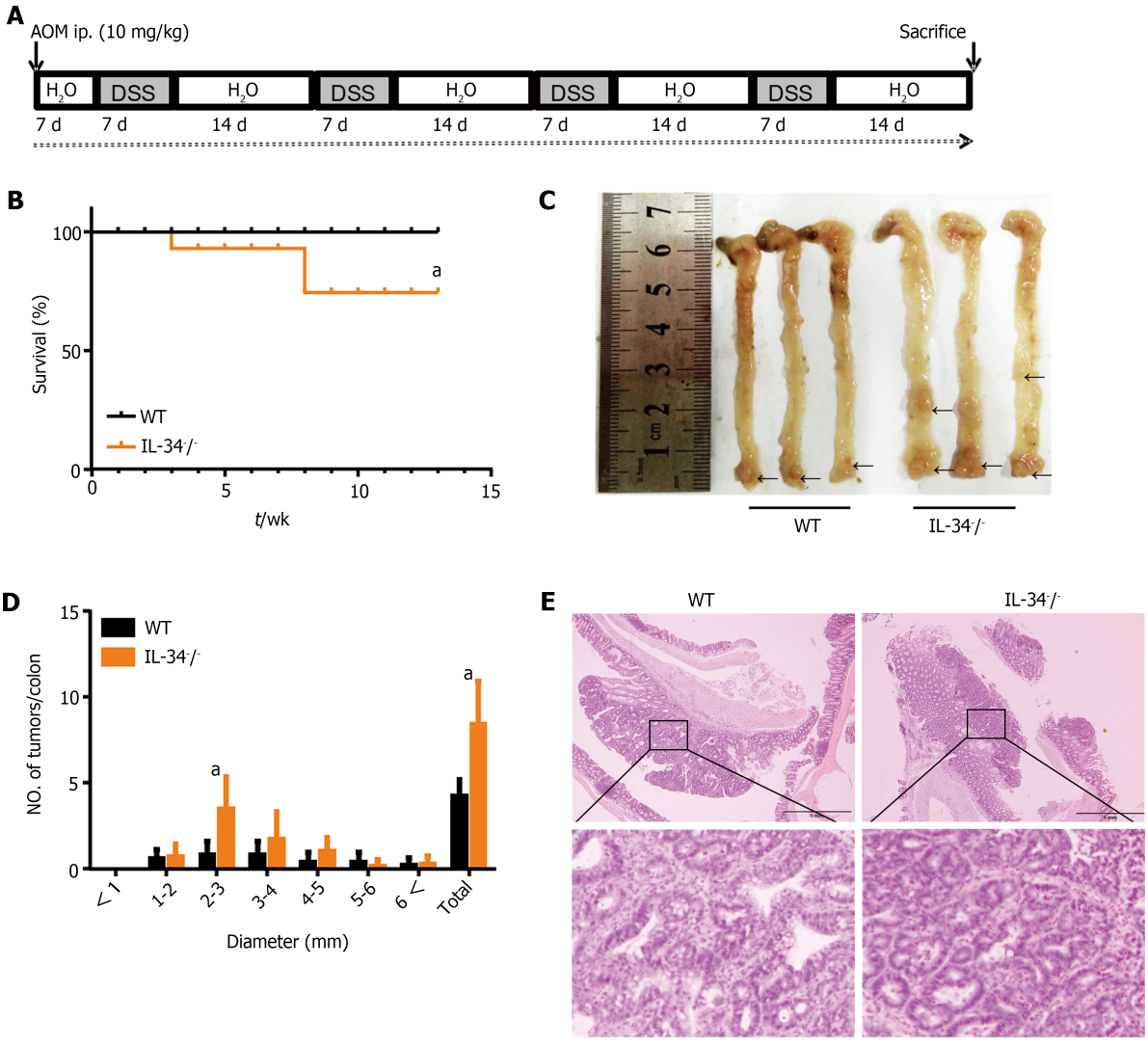

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Figure 7 Interleukin-34 deficiency promotes azoxymethane /dextran-sodium-sulfate-induced colitis-associated tumorigenesis. A: Experimental design was shown as diagram. WT and Interleukin-34 (IL-34)$^{-/-}$ mice were given a single injection of azoxymethane (AOM, 10 mg/kg). Seven days later, mice were treated with 1.25% dextran sodium sulfate (DSS) (w/v) in drinking water for seven consecutive days, and given fresh drinking water for 14 d. Seven days of DSS and 14 d of fresh water as a cycle, repeated four times; B: Kaplan-Meier survival curves were plotted in IL-34$^{-/-}$ and WT mice treated with AOM/DSS (n = 10 per group); C and D: Representative photo of colon (C) and tumor number in colon (D) in IL-34$^{-/-}$ and WT mice treated with AOM/DSS (n = 10 per group); E: Representative hematoxylin and eosin-stained colon sections in IL-34$^{-/-}$ and WT mice treated with AOM/DSS (n = 10 per group). AOM: Azoxymethane; DSS: Dextran sodium sulfate; WT: Wild type. Bars: 1 mm (top) and 100 μm (bottom). Data depict mean ± SD. $^{a}P < 0.05$.

tissue remodeling[17]. Regulation of M1 to M2 phenotype switch has been confirmed to promote the proliferative phase of wound healing and ameliorates DSS-induced colitis, indicating a possible role of IL-34 in alleviation of colonic inflammation[17,18]. In our study, IL-34-deficient mice treated with DSS presented with worse colitis with significant accumulation of macrophages compared to DSS-treated wild-type control mice. It is speculated that downregulation of the M1 to M2 macrophage phenotypic switch results in more severe colitis. However, M2/M1 macrophage ratio was not significantly decreased in DSS-treated IL-34-deficient mice. We subsequently depleted the macrophages in the murine colitis model by administration of Clo-lips, which is a well-established method for macrophage

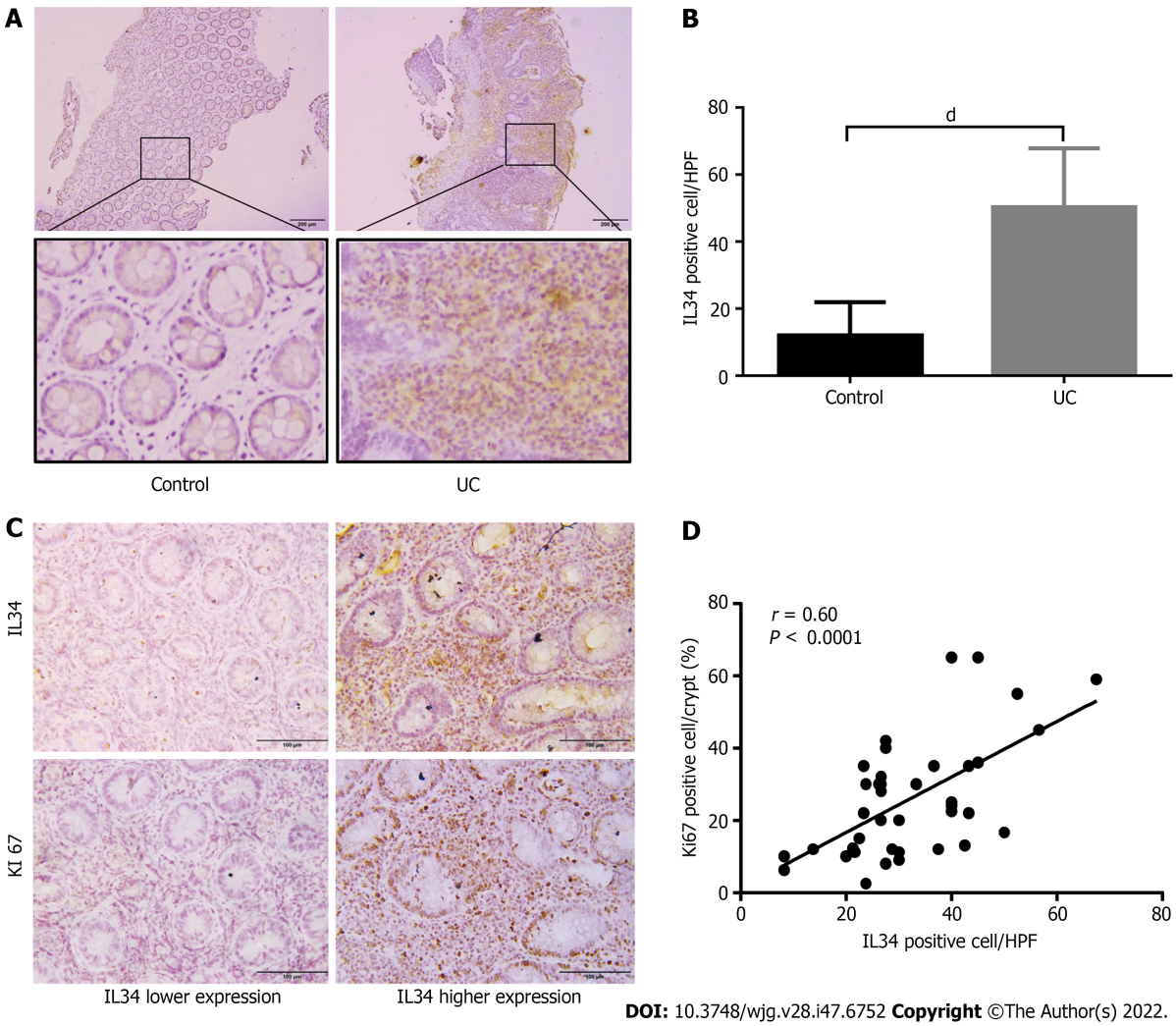

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Figure 8 Interleukin-34 expression was elevated in inflamed mucosa and associated with colonic epithelium proliferation in active ulcerative colitis. A: Representative microscopic pictures of interleukin-34 (IL-34) immunohistochemical staining in colonic biopsies of healthy controls or individuals with active ulcerative colitis (UC); B: Percentage of IL-34-positive cells per high-power field were quantified in colonic biopsies of healthy controls (n = 20) and active UC patients (n = 40); C: Representative photomicrographs of immunostaining of Ki67 in colonic biopsies of active UC patients in IL-34 Lower expression and higher expression groups; D: Correlation analysis of colonic IL-34 expression and Ki 67-positive cells in active UC patients. IL-34: Interleukin-34; UC: Ulcerative colitis. Scale bar indicates 100 μm or 200 μm. $^{d}P < 0.001$.

depletion[30], showing that IL-34 deficiency aggravated murine colitis whether or not macrophages were depleted. Our results suggested that the protective effect of IL-34 on experimental colitis was not primarily dependent on macrophage polarization.

Mucosal healing largely relies on controlled colonic epithelial proliferation and apoptosis, which are manipulated by multiple growth factors, gut peptides, cytokines, as well as signaling pathways[25]. Given different DSS-induced injury levels in normal and IL-34-deficient colonic mucosa, we should be cautious to draw the conclusion that IL-34 deficiency inhibits the mucosal healing process *in vivo*. Based upon immunohistochemical staining, IL-34 was predominantly localized in the colon stromal tissue in our study. The specific cellular source of IL-34 needs to be further explored. In the acute colitis model, epithelial cell proliferation was inhibited but epithelial cell apoptosis was induced in IL-34$^{-/-}$ mice. CSF-1R was compensatorily increased in the IL-34$^{-/-}$ mice treated with DSS. Furthermore, it has been proved that IL-34 expression is positively correlated with epithelial proliferation in mucosal biopsies from UC patients. No correlation was shown between IL-34 expression level and apoptosis in UC patients. It is speculated that IL-34 might be produced from colon stromal tissue and promote colonic epithelial cell growth and wound closure by binding to CSF-1R in inflammatory conditions. Further studies are warranted to investigate the potential function of IL-34 in colonic epithelial cells.

Emerging data have demonstrated the multidimensional role of IL-34 in tumor progression and metastasis. The function of IL-34 in cancer may vary among different types of tumors[31]. A recent study on the relationship between IL-34 and gastric cancer proposed that the reduction of IL-34 in gastric cancer was inversely related to the degree of tumor differentiation and was closely related to the poor survival rate of patients[32]. However, the relationship of IL-34 to colorectal cancer remains contro-

versial. It has been recently reported that IL-34 stimulates colorectal adenocarcinoma cell proliferation *via* an ERK1/2-dependent pathway. The connection between chronic colon inflammation and colitis-associated cancer has been documented in UC patients. Repeated mucosal damage and repair in the inflamed microenvironment can result in uncontrolled epithelial cell proliferation and induce cancer events[33]. In a cohort study, Wang *et al*[34] showed that low expression of IL-34 is associated with poor survival in colorectal cancer. Accumulating evidence suggests that chronic inflammation enhances the development of colitis-associated cancer through multiple mechanisms, including oxidative stress, DNA damage, abnormal immune response, and gut microbiome dysbiosis[35]. In our study, tumor burden was markedly enhanced in IL-34$^{-/-}$ mice treated with AOM/DSS compared to wild-type control mice. We assumed that IL-34 deficiency worsened the colonic inflammation response and tissue damage, and thus sustained inflammatory injury increased the risk of colitis-associated cancer.

CONCLUSION

In summary, our work has highlighted the role of IL-34 during acute experimental colitis and wound healing. We found that IL-34 does not drive the inflammatory response and tissue destruction under physiological conditions, but protects the host from inflammatory injury and reduces the risk of colitis-associated cancer. IL-34 might serve as a potential therapeutic target for inducing mucosal healing in treatment for UC and reducing colitis-associated cancer in UC.

ARTICLE HIGHLIGHTS

Research background

The exact pathogenesis of ulcerative colitis (UC) remains unclear. Identification of distinctive cytokines involved in immunopathogenesis of UC has become a hot spot for the development of biological therapies. The expression of interleukin (IL)-34 is upregulated in active UC but the molecular function and underlying mechanism are largely unknown.

Research motivation

Whether IL-34 is a "friend or foe" in the pathogenesis of UC remains be explored. In this study, we investigated the potential role of IL-34 in experimental colitis, colitis-associated carcinogenesis and UC.

Research objectives

To investigate the function of IL-34 in acute colitis, in a wound healing model and in colitis-associated cancer, and the IL-34 expression pattern and its relationship with colonic epithelium proliferation in active UC patients.

Research methods

We conducted a controlled study using IL-34 knockout mice and wild-type mice (C57BL/6J). Colitis was induced by administration of dextran sodium sulfate, and carcinogenesis was induced by azoxymethane. Whether the impact of IL-34 on colitis was dependent on macrophages was validated by depletion of macrophages in a murine model. The association between IL-34 expression and epithelial cell proliferation was determined in patients with active UC.

Research results

IL-34 deficiency *in vivo* exacerbated colitis in mice during acute and wound healing phases and increased tumor susceptibility in the mouse colon. The effect was independent of macrophage differentiation and polarization. IL-34 was markedly increased in patients with active UC and the expression was positively correlated with epithelial cell proliferation in UC.

Research conclusions

IL-34 deficiency exacerbates colonic inflammation and accelerates colitis-associated carcinogenesis in mice.

Research perspectives

IL-34 might serve as a potential therapeutic target for inducing mucosal healing in treatment of UC and reducing colitis-associated cancer in UC.

FOOTNOTES

Author contributions: Liu ZX performed the main part of the study; Chen WJ and Wang Y performed the main part of the study and wrote original draft; Liu YC, Cheng TC, Luo LL, Chen L, and Ju LL provided technical support and analyzed the data; Chen BQ, Liu Y, Li M, and Feng N contributed to part of the experiments; Shao JG designed the study and guided the manuscript writing; Bian ZL Conceived and designed the study; Liu ZX, Chen WJ, and Wang Y contributed equally to this work; all authors have read and approve the final manuscript.

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Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the Institutional Ethics Committee of Nantong University (No: S20151221-908).

Informed consent statement: All patients and healthy people signed an informed consent form.

Conflict-of-interest statement: All authors confirm that there are no conflicts of interest.

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Basic Study

Dickkopf-related protein 1/cytoskeleton-associated protein 4 signaling activation by *Helicobacter pylori*-induced activator protein-1 promotes gastric tumorigenesis *via* the PI3K/AKT/mTOR pathway

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Abstract

BACKGROUND

Gastric cancer (GC) is a common malignant tumor with high incidence and mortality rates globally, especially in East Asian countries. *Helicobacter pylori* (*H. pylori*) infection is a significant and independent risk factor for GC. However, its underlying mechanism of action is not fully understood. Dickkopf-related protein (DKK) 1 is a Wnt signaling antagonist, and cytoskeleton-associated protein (CKAP) 4 is a newly identified DKK1 receptor. Recent studies found that the binding of DKK1 to CAKP4 mediated the procancer signaling of DKK1 independent of Wnt signaling. We hypothesize that *H. pylori*-induced activation of DKK1/CKAP4 signaling contributes to the initiation and progression of GC.

AIM

To investigate the interaction of *H. pylori* infection, DKK1 and CAKP4 in GC, as well as the underlying molecular mechanisms.

METHODS

RNA sequencing was used to identify differentially expressed genes (DEGs) between *H. pylori*-infected and uninfected primary GC cells. Gain- and loss-of-

function experiments were performed to verify the *H. pylori*-induced upregulation of activator protein-1 (AP-1) in GC cells. A dual-luciferase reporter assay and co-immunoprecipitation were used to determine the binding of AP-1 to the DKK1 promoter and DKK1 to CKAP4. Western blotting and immunohistochemistry detected the expression of DKK1, CKAP4, and phosphatidylinositol 3-kinase (PI3K) pathway-related proteins in GC cells and tissues. Functional experiments and tumorigenicity in nude mice detected malignant behavior of GC cells *in vitro* and *in vivo*.

RESULTS

We identified 32 DEGs between primary GC cells with and without *H. pylori* infection, including JUN, fos-like antigen-1 (FOSL1), and DKK1, and confirmed that the three proteins and CKAP4 were highly expressed in *H. pylori*-infected GC cells, *H. pylori*-infected gerbil gastric tissues, and human GC tissues. JUN and FOSL1 form AP-1 to transcriptionally activate DKK1 expression by binding to the DKK1 promoter. Activated DKK1 bound to CKAP4, but not the most common Wnt coreceptor low-density lipoprotein receptor-related protein 5/6, to promote GC cell growth, colony formation, migration, invasion, and xenograft tumor growth in nude mice. All these effects were driven by activation of the PI3K/AKT/mammalian target of rapamycin (mTOR) pathway. Targeting the PI3K signaling pathway by LY294002 inhibited DKK1-mediated CKAP4/PI3K signaling activity and the malignant behavior of GC cells.

CONCLUSION

H. pylori induces JUN and FOSL1 expression to form AP-1, which transcriptionally activates DKK1. Binding of DKK1 to KAKP4 contributes to gastric tumorigenesis *via* the PI3K/AKT/mTOR pathway.

Key Words: Gastric cancer; *Helicobacter pylori*; Dickkopf-related protein 1; Cytoskeleton-associated protein 4; Phosphatidylinositol 3-kinase pathway

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Core Tip: *Helicobacter pylori* (*H. pylori*) infection is the most significant risk factor for gastric cancer (GC). More than half of the global population has *H. pylori* infection, and 1%-3% of the infected individuals develop GC, but the mechanism behind this link remains unclear. Here, we identified 32 highly expressed genes in *H. pylori*-infected GC cells and demonstrated that *H. pylori*-induced high expression of JUN and fos-like antigen-1 formed activator protein-1 to transcriptionally activate dickkopf-related protein (DKK) 1, which by binding to cytoskeleton-associated protein 4 (CKAP4) receptor activated the PI3K/AKT/mammalian target of rapamycin pathway and, consequently, gastric tumorigenesis. Targeting the DKK1/CKAP4 interaction may be a novel strategy to treat GC.

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INTRODUCTION

Gastric cancer (GC) is the fifth most prevalent tumor and fourth leading cause of tumor-related mortality worldwide[1]. Although the incidence and mortality rates of this malignancy have steadily decreased over the past several decades, GC remains a significant health problem in developing nations [2]. Most patients with GC present at an advanced stage when diagnosed, and metastasis and recurrence in individuals are commonly noted[3]. *Helicobacter pylori* (*H. pylori*) are Gram-negative and microaerophilic pathogenic bacteria. *H. pylori* infection is the most significant risk factor for GC. The International Agency for Research on Cancer designated it a Group I carcinogen for GC in 1994[4]. More than half of the global population has *H. pylori* infection, and 1%-3% of infected individuals develop GC [5]. Globally, an estimated 89.4% and 20% of new noncardia and cardia GC cases were attributable to *H. pylori* infection in 2018[6]. However, this figure was 78.5% for noncardia GC and 62.1% for cardia GC in China[7]. Although many studies have investigated the link between *H. pylori* infection and GC[8], the

molecular mechanisms are not fully understood.

By RNA sequencing, we discovered that JUN (also known as c-JUN), fos-like antigen-1 (FOSL1, a FOS family member encoding FRA-1), and Dickkopf-related protein (DKK) 1 were elevated in primary GC cells infected with *H. pylori*. JUN and FOSL1 are the transcription factor activator protein (AP)-1 complex components and form the JUN::JUN homodimer and the JUN::FOSL1 heterodimer to regulate target gene transcription *via* binding to the promoters and enhancers of target genes[9]. *In vitro* and *in vivo* studies showed that activator protein-1 (AP-1) controlled tumor growth, progression and drug resistance[10,11]. DKK1 is a potent antagonist that suppresses oncogenic Wnt/β-catenin signaling and tumors by binding to the Wnt coreceptor: low-density lipoprotein receptor-related protein (LRP) 5/6[12,13]. However, recent studies demonstrated that DKK1 behaved like an oncogene in a variety of cancers [14,15]. Several studies consistently indicated that DKK1 levels were elevated in GC patient cancer tissues and serum, and higher DKK1 levels were significantly associated with worse outcomes[16-18]. However, little is known about the transcriptional regulation of DKK1, its relationship with *H. pylori*, and the mechanism of DKK1 promotion of gastric carcinogenesis.

Cytoskeleton-associated protein (CKAP) 4 is a newly identified DKK1 receptor[19]. The binding of DKK1 to CKAP4 recruits phosphatidylinositol 3-kinase (PI3K) *via* association between the proline-rich region of CKAP4 and the Src homology 3 domain of PI3K, which leads to the activation of serine/threonine-protein kinase AKT[20]. Although there is no evidence that the DKK1/CKAP4 axis exists in GC, the PI3K pathway involved in the malignant transformation of cells is activated in most GC patients[21]. AKT and p-AKT were overexpressed in > 74% of GC patients[22], and phosphorylated mammalian target of rapamycin (p-mTOR) expression was found in 60% of gastric adenocarcinoma specimens[23]. Therefore, we postulate that DKK1 contributes to GC through the PI3K pathway but not the Wnt pathway.

Here, we revealed that *H. pylori* infection induced high expression of JUN and FOSL1, which formed AP-1 to activate DKK1 transcriptionally. DKK1 binding to CKAP4, but not the most common Wnt coreceptor LRP5/6, promoted GC growth and invasion by triggering the PI3K/AKT/mTOR pathway. The results provide novel insight into the molecular mechanism underlying *H. pylori*-induced gastric carcinogenesis.

MATERIALS AND METHODS

H. pylori strains and cell lines

H. pylori GZ7 is a typical East Asian strain (*cagA*$^+$) that was isolated from the gastric mucosa of a GC patient by our group[24]. *H. pylori* 26695 (ATCC 700392) is a typical western strain (*cagA*$^+$) that was purchased from the American Type Culture Collection (ATCC, Manassas, VA, United States). Two strains were cultured on Columbia agar plates supplemented with *H. pylori* selective supplement (Oxoid, United Kingdom) and 10% sheep blood at 37 °C in a microaerobic environment (5% O_2, 10% CO_2, 85% N_2).

The human GC cell lines AGS, NCI-N87 and SNU-16 cells were purchased from ATCC, and SGC-7901 and BGC823 cells were obtained from the tissue bank in Shanghai, China. Primary GC cells were separated and identified in our laboratory[25]. All cell lines were grown in 5% CO2 at 37 °C in RPMI-1640 with 10% FCS (Gibco, United States).

Human GC tissues

From June to December 2021, 12 pairs of GC and paracancer normal tissues were collected during surgery at the Affiliated Hospital of Guizhou Medical University in China. Tissues were immediately formalin-fixed and paraffin-embedded for pathological evaluation and immunohistochemistry. All subjects provided their written informed consent. The study was performed following the Declaration of Helsinki. The Guizhou Medical University Ethics Committee approved the study protocol, No. 2017(43).

Mongolian gerbil stomach tissues

In our earlier work, Mongolian gerbils were intragastrically infected with *H. pylori* NCTC 11637 (ATCC 43504, *cagA*$^+$) for 2 years. At 3-24 mo after infection, gerbils developed erosion, atrophy, intestinal metaplasia, and well-differentiated GC in the gastric mucosa[26]. The stomach tissues of gerbils (n = 3 at each timepoint) were removed after decapitation and immediately fixed and embedded. The embedded tissues were sliced into sections (5 μm thick) and stained for immunohistochemistry.

RNA-sequencing

Three primary GC cell lines (5 × 10^7) were cultured with 50 multiplicity of infection (MOI) *H. pylori* GZ7 for 6 h before being harvested. TRIzol (Invitrogen, Carlsbad, CA, United States) was used to extract overall RNA, which was then purified by DNase I treatment. After being enriched by Oligo (dT) beads, eukaryotic mRNA was interrupted into short fragments and synthesized into cDNA. Using the Illumina

TruSeq library construction kit, purified cDNA was amplified by PCR to construct the RNA-sequencing (RNA-seq) library. After the library was tested for quality using the Agilent 2100 Bioanalyzer and ABI StepOnePlus Real-Time PCR System, the library was sequenced on the Illumina HiSeqX10 platform by Beijing Genomics Institute (China). Sequence reads were mapped to the reference human genome (hg19), and the gene and transcript expression levels were calculated. Differentially expressed genes (DEGs) with a fold-change > 2 and false discovery rate (FDR) < 0.05 were identified between *H. pylori*-infected and uninfected cells. The DEGs shared by three datasets were selected for further bioinformatics analysis. The R heatmap package (version 0.7.7) was used to generate a heatmap. Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and protein-protein interaction (PPI) analyses were performed using the Gene Ontology Consortium (http://geneontology.org/), KOBAS (http://kobas.cbi.pku.edu.cn/), and the STRING tool (https://string-db.org/), respectively. KEGG pathways and PPI networks were visualized using Cytoscape software (version 3.7.2). Gene set enrichment analysis (GSEA) was carried out using GSEA software (version 4.1.0). A significant gene set was defined as having an FDR < 0.25 and $P < 0.01$.

Dual-luciferase reporter assay

The expression vectors of JUN and FOSL1 were purchased from Hanheng Biotech (Shanghai, China). The human DKK1 promoter region from -1100 to + 1 bp was cloned into the luciferase reporter vector pGL4.29 (Promega, Charbonnières-les-Bains, France), which was cotransfected with pcDNA3.1-JUN and/or pcDNA3.1-FOSL1 into AGS cells for 48 h using Lipofectamine 2000 (Invitrogen). The activities of firefly luciferase and Renilla luciferase were determined using the dual luciferase reporter system (Promega). The ratio of firefly luciferase activities to Renilla luciferase activities (Fluc/Rluc) was used to describe the reporter activities.

Western blotting

Proteins (25 g) were resolved *via* SDS-PAGE and transferred by electroblotting onto the PVDF membrane (Millipore, Billerica, MA, United States). After blocking with 5% nonfat milk, primary antibodies were used to probe the membrane overnight at 4 °C. The membrane was incubated with horseradish-peroxidase-conjugated secondary antibodies. The bands were visualized using electro-chemiluminescence. Details of the antibodies are provided in Supplementary Table 1.

RT-qPCR

RNA isolation was performed by TRIzol reagent. cDNA was synthesized through the process of reverse transcription (RT) using a standard protocol according to the manufacturer's instructions. Quantitative PCR (qPCR) was performed using fluorogenic SYBR Green (BioRad, Hercules, CA, United States). The $2^{-\Delta\Delta CT}$ method was used for relative mRNA quantitation of target genes. GAPDH gene served as the loading control. RT-qPCR primers are listed in Supplemental Table 2.

Immunohistochemistry and immunofluorescence

Gerbil stomachs, nude mouse xenografts, and human GC tissues were cut into 5-μm thick sections. Immunohistochemistry was used to detect expression of JUN, DKK1, CKAP4 and Ki67 in these tissues, and immunofluorescence imaging was used to assess β-catenin expression and nuclear translocation in DKK1-silenced AGS cells, as described previously[25]. The quantification of immunohistochemical staining was performed by Image J software. Immunohistochemistry score was determined by multiplying the intensity score and the percentage score[27]. Details of the antibodies are provided in Supplementary Table 1.

Cell growth curve

Cell viability was determined using Cell Counting Kit-8 kit (CCK-8, Dojindo, Japan) in accordance with the kit instructions. Cells (1000 cells per well) were placed in a 96-well plate in sextuplicate per condition and cultured for 1-6 d. CCK-8 solution (10 μL) was added to each well at the corresponding time points, and the cells were cultured for an additional 2 h. CCK-8 was used to detect cell viability.

Colony formation assay

Cells (500 cells per well) were placed in a 6-well plate in triplicate per condition and cultured for 2 wk. The growth medium was changed once every 4 d. Two weeks later, 0.1% crystal violet (Solarbio, China) was used to stain the colonies after being fixed with 4% paraformaldehyde. Colony count was performed using a microscope.

Transwell assay

Transwell migration and invasion assays were performed using a 24-well Transwell insert (pore size of 8 μm) with and without Matrigel (Biosciences, San Jose, CA, United States). A total of 1×10^4 cells in medium with 1% FBS was added to the upper chamber of the Transwell, and 800 μL of medium with 10% FBS was added in lower chamber. At 24 h and 72 h after culture, 4% paraformaldehyde was used to fix the inserts for 30 min at room temperature before being stained in a 0.1% crystal violet solution and

imaged. Cells that migrated or invaded to the lower chamber were counted using a microscope.

Cell cycle and apoptosis

Cell cycle and apoptosis analyses were performed using flow cytometry, as previously described[25]. Cell cycle distribution and apoptosis were analyzed using FloJo software.

Co-immunoprecipitation

Co-immunoprecipitation (Co-IP) was performed using an Absin Co-IP kit (Shanghai, China). BGC823 cells (3×10^6) with or without *H. pylori* infection were sonicated three times for 20 s each in ice-cold lysis buffer before being centrifuged at 14000 × g for 10 min at 4 °C. The supernatant was then collected as whole-cell lysates. Cell lysates were incubated overnight at 4 °C with a CKAP4 antibody (2 μg) and control IgG (1 μg). Protein A- and protein G-Sepharose (10 μL) (Sigma-Aldrich, St. Louis, MO, United States) were added to the cell lysates and left on for 8 h at 4 °C. After 1 min of centrifugation at 12000 × g, the beads were collected and washed three times with wash buffer. The beads were boiled in 2 × Laemmeli sample buffer for 5 min and centrifugated for 1 min at 14000 × g and 4 °C. SDS-PAGE was used to resolve proteins in the supernatants (15 μL) after being collected. Western blotting was used to detect DKK1 by an anti-DKK1 antibody (1:400).

Tumorigenicity in nude mice

The ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines (https://www.nc3rs.org.uk/arrive-guidelines) were followed for all animal experiments to minimize pain and discomfort for the animals. Guizhou Medical University Animal Care Welfare Committee approved the animal study (No. 1702155). Ten BALB/c nude mice (male, age 3-4 wk) were provided by Chongqing Tengxin Biotechnology (Chongqing, China). Mice were accustomed to the laboratory environment that was a 12-h light/12-h dark cycle at 23 °C with 50% humidity and were given water and food *ad libitum*. After 2 wk, BGC823 cells (2×10^6) stably overexpressing DKK1 and negative control cells were transplanted subcutaneously into the flanks of the mice (5 mice per group), and the tumor size was measured every 3 d. After 16 d, all mice were killed by intravenous injection of barbiturate overdose (150 mg/kg), and the tumors were removed. Tumor weights and volumes were determined using the formula (length × width^2)/2.

Lentivirus infection

The lentiviruses containing shJUN and shDKK1, overexpression lentiviruses for JUN and DKK1, and control lentiviruses were obtained from Shanghai Jikai Gene Co. Ltd. (China). These lentiviruses were used to infect AGS, SGC-7901, NCT-N87, SNU-16 and BGC823 cells (2×10^5) in a 12-well plate at MOI 10-30 for 48 h. Polybrene (8 μg/mL; Sigma) was added to the 12-well plate to increase the efficiency of lentivirus infection. After 24 h, the medium containing lentiviruses and polybrene was changed with fresh medium and cultured for a further 48 h. After that, the stable cell lines were selected for 2 wk using 2 μg/mL puromycin, in which the medium containing puromycin was changed every 2-3 d. The cells were maintained in 1 μg/mL puromycin. Western blotting and RT-qPCR were used to ensure that target genes were stably knocked down or overexpressed.

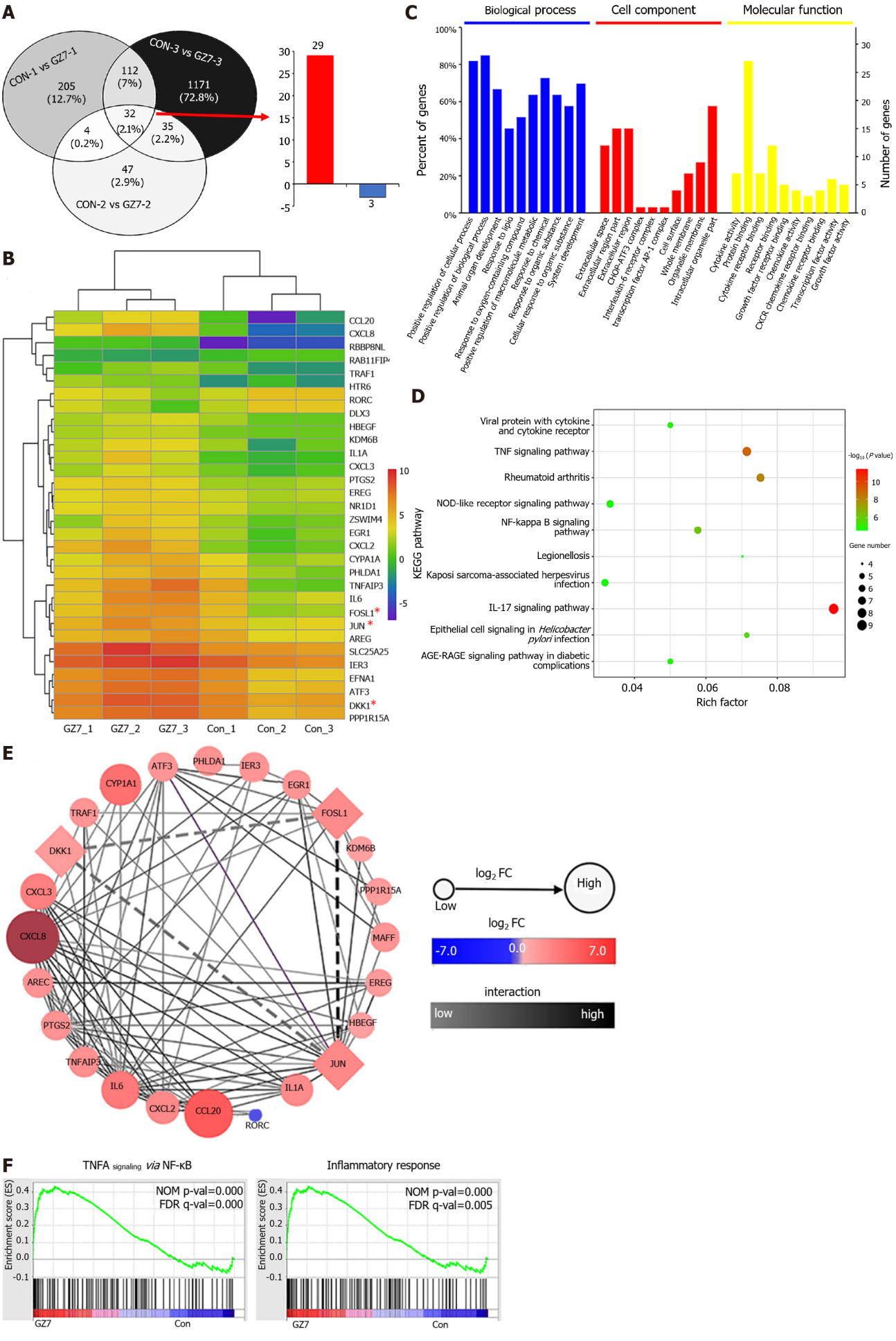
Statistical analysis

SPSS 22.0 (IBM, Armonk, NY, United States) was used for all statistical analyses. ImageJ software (National Institutes of Health, Bethesda, MD, United States) was used for the quantification of images from immunohistochemistry. GraphPad Prism 8.0 (La Jolla, CA, United States) was used to create statistical figures. All presented images were representative of three or more individual experiments. One-way or two-way analysis of variance was performed for comparisons between multiple groups. All results were expressed as mean ± SD. $P < 0.05$ indicated statistically significant differences.

RESULTS

Analysis of DEGs between primary GC cells with and without H. pylori infection

RNA-seq analysis identified 32 DEGs, including 29 upregulated and three downregulated genes that were shared by three pairs of primary GC cells infected with or without *H. pylori* (Figure 1A and B). These DEGs were mostly enriched in the regulation of biological processes, cytokine activity, protein binding, and tumor necrosis factor (TNF), nuclear factor (NF)-κB, and interleukin (IL)-17 pathways according to GO and KEGG pathway enrichment analysis (Figure 1C and D). The PPI network identified the hub genes *JUN*, *CXCL8*, *CCL20*, and *FOSL1* and their interactions, and JUN and FOLS1 were directly connected to DKK1 (Figure 1E). Based on the GSEA of DEGs, TNFA signaling *via* NF-κB, the inflammatory response, apoptosis, hypoxia, MYC targets, and the P53 pathway were enriched in *H. pylori*-infected GC cells (Figure 1F). These molecular events are closely associated with GC development and progression. JUN, FOLS1, and DKK1 were selected for further investigation.


A
CON-1 vs GZ7-1
CON-3 vs GZ7-3
CON-2 vs GZ7-2
205 (12.7%)
112 (7%)
1171 (72.8%)
32 (2.1%)
4 (0.2%)
35 (2.2%)
47 (2.9%)
29
3
B
CCL20
CXCL8
RBBP8NL
RAB11FIP4
TRAF1
HTR6
RORC
DLX3
HBEGF
KDM6B
IL1A
CXCL3
PTGS2
EREG
NR1D1
ZSWIM4
EGR1
CXCL2
CYPA1A
PHLDA1
TNFAIP3
IL6
FOSL1*
JUN*
AREG
SLC25A25
IER3
EFNA1
ATF3
DKK1*
PPP1R15A
GZ7_1
GZ7_2
GZ7_3
Con_1
Con_2
Con_3
C
Biological process
Cell component
Molecular function
Percent of genes
Number of genes
Positive regulation of cellular process
Positive regulation of biological process
Animal organ development
Response to lipid
Response to oxygen-containing compound
Positive regulation of macromolecule metabolic
Response to chemical
Response to organic substance
Cellular response to organic substance
System development
Extracellular space
Extracellular region part
Extracellular region
CHOP-ATF3 complex
Interleukin-6 receptor complex
transcription factor AP-1 complex
Cell surface
Whole membrane
Organelle membrane
Intracellular organelle part
Cytokine activity
Protein binding
Cytokine receptor binding
Receptor binding
Growth factor receptor binding
Chemokine activity
CXCR chemokine receptor binding
Chemokine receptor binding
Transcription factor activity
Growth factor activity
D
KEGG pathway
Viral protein with cytokine and cytokine receptor
TNF signaling pathway
Rheumatoid arthritis
NOD-like receptor signaling pathway
NF-kappa B signaling pathway
Legionellosis
Kaposi sarcoma-associated herpesvirus infection
IL-17 signaling pathway
Epithelial cell signaling in Helicobacter pylori infection
AGE-RAGE signaling pathway in diabetic complications
Rich factor
-log10 (P value)
Gene number
E
ATF3
PHLDA1
IER3
CYP1A1
EGR1
TRAF1
FOSL1
DKK1
KDM6B
CXCL3
PPP1R15A
CXCL8
MAFF
AREC
EREG
PTGS2
HBEGF
TNFAIP3
JUN
IL6
IL1A
CXCL2
CCL20
RORC
log2 FC
Low
High
-7.0
0.0
7.0
interaction
low
high
F
TNFA signaling via NF-κB
NOM p-val=0.000
FDR q-val=0.000
Inflammatory response
NOM p-val=0.000
FDR q-val=0.005
Enrichment score (ES)
GZ7
Con

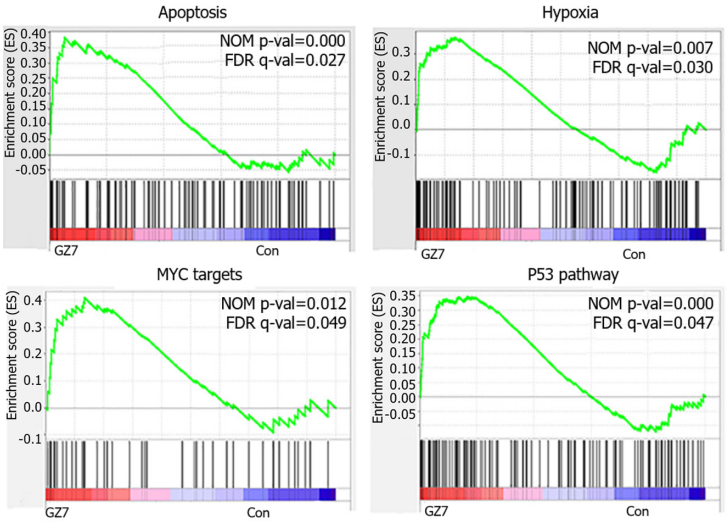


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Figure 1 Comparison of differentially expressed genes between primary gastric cancer cells with and without *Helicobacter pylori* infection. A: Venn diagram of differentially expressed genes (>DEGs); B: DEGs heatmap analysis by R heatmap package. Asterisks indicate the genes investigated in this study; C: Gene Ontology analysis of the DEGs; D: Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis of the DEGs; E: Protein-protein interaction network of the DEGs; F: Gene set enrichment analysis (GSEA) enrichment analyses of the DEGs with GSEA version 4.1.0. Con: Control; GZ7: *Helicobacter pylori* GZ7. TNF: Tumor necrosis factor; FOSL1: Fos-like antigen-1; DKK1: Dickkopf-related protein 1.

H. pylori infection induces JUN and FOSL1 upregulation to activate DKK1 expression via binding to the DKK1 promoter

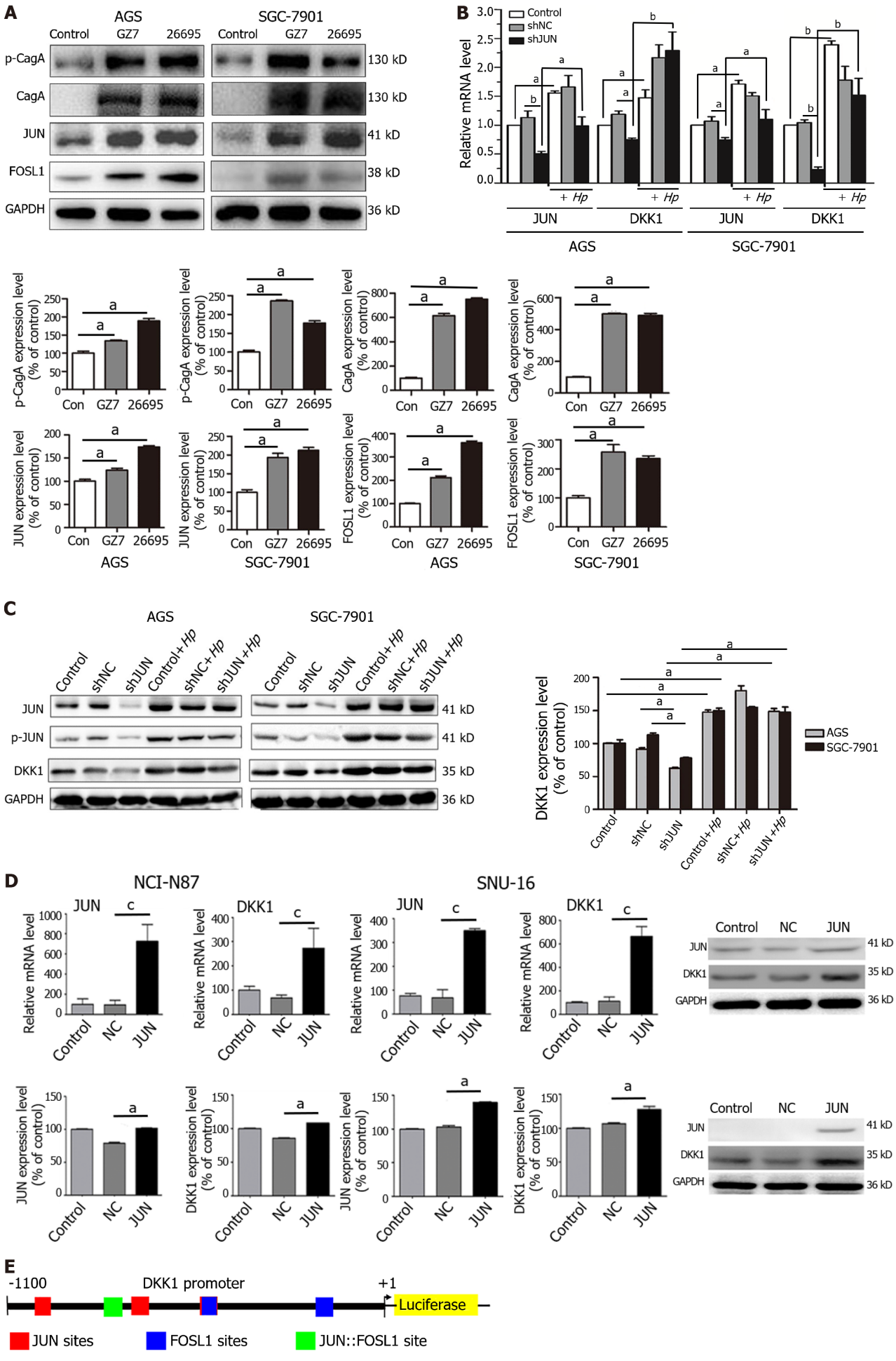
To confirm the results of RNA-seq, AGS and SGC-7901 cells were infected with *H. pylori* GZ7 and 26695. We discovered that JUN and FOSL1 proteins were highly expressed in *H. pylori*-infected cells compared to the control cells, which supported the RNA-seq resultss (Figure 2A). The *H. pylori* virulence factor CagA and its phosphorylation levels were dramatically elevated in *H. pylori*-infected cells (Figure 2A).

We determined JUN expression in eight GC cell lines. JUN was highly expressed in AGS and SGC-7901 cells but weakly expressed in NCI-N87 and SNU-16 cells (Supplementary Figure 1). JUN was knocked down using short hairpin RNA (shRNA) in AGS and SGC-7901 cells and overexpressed using lentivirus infection in NCI-N87 and SNU-16 cells. RT-qPCR and western blotting revealed a substantial decrease in DKK1 expression after JUN knockdown. When the JUN-knockdown cells were infected with *H. pylori*, the mRNA and protein levels of DKK1 recovered and JUN expression and phosphorylation increased (Figure 2B and C). In contrast, the mRNA and protein level of DKK1 was significantly increased in SNU-16 and NCI-N87 cells after JUN overexpression (Figure 2D). These findings revealed that *H. pylori*-induced JUN enhanced DKK1 expression.

JUN and FOSL1 comprise AP-1 *via* their basic leucine zipper domain[28]. AP-1-binding sites include the 5′-TGA(C/G) TCA-3′ (TRE motif), 5′-TCACGTCA-3′ (CRE motif), and their single-base variants[29]. To confirm that AP-1 transcriptionally regulated DKK1 expression, we predicted AP-1-binding sites in the promoter region from -1100 to + 1 bp upstream of the DKK1 transcription site, which is highly conserved in mammalian genomic DNA[30]. Three JUN sites, two FOSL1 sites, and a JUN::FOSL site were observed in the DKK1 promoter (Figure 2E). The DKK1 promoter region from -1100 to + 1 bp was inserted into a luciferase reporter plasmid, which was cotransfected into AGS cells with JUN and/or FOSL1 vectors. The dual-luciferase reporter assay indicated that transfection of AGS cells with JUN and FOSL1 markedly increased the luciferase activity, and the effect of the JUN vector was stronger. JUN and FOSL1 cotransfection exhibited the highest luciferase activity in AGS cells (Figure 2F). This result confirmed that AP-1, including the JUN::JUN and JUN::FOSL1 complexes, bound to the DKK1 promoter to initiate DKK1 transcription.

High JUN and DKK1 expression in gerbil gastric tissues infected with H. pylori and human GC tissues

We successfully established GC models in Mongolian gerbils *via H. pylori* infection in a previous study, in which erosion, atrophy, intestinal metaplasia, and well-differentiated GC were progressively detected in the stomach mucosa[26]. Positive JUN and DKK1 staining increased progressively in gerbil gastric epithelium with *H. pylori* infection from 3 mo to 24 mo in the present study (Figure 3A), which suggested that the expression of JUN and DKK1 was linked to the pathological development of GC in gerbils. Human GC genomic data were extracted from the TCGA-Stomach Adenocarcinoma database (TCGA-STAD), which included 375 cancer and 391 normal tissues. The expression of DKK1 mRNA was


A
AGS
SGC-7901
Control
GZ7
26695
p-CagA
CagA
JUN
FOSL1
GAPDH
130 kD
130 kD
41 kD
38 kD
36 kD
B
Control
shNC
shJUN
Relative mRNA level
+ Hp
JUN
DKK1
AGS
SGC-7901
p-CagA expression level (% of control)
CagA expression level (% of control)
JUN expression level (% of control)
FOSL1 expression level (% of control)
Con
C
Control
shNC
shJUN
Control+Hp
shNC+Hp
shJUN+Hp
p-JUN
DKK1
41 kD
35 kD
DKK1 expression level (% of control)
D
NCI-N87
SNU-16
Relative mRNA level
NC
E
-1100
DKK1 promoter
+1
Luciferase
JUN sites
FOSL1 sites
JUN::FOSL1 site

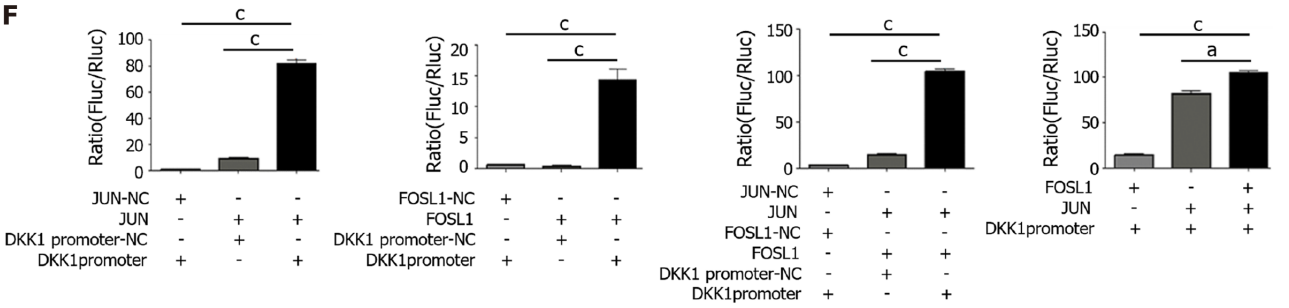


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Figure 2 *Helicobacter pylori* infection increases JUN and fos-like antigen-1 expression to activate the dickkopf-related protein 1 promoter. A: Western blotting for CagA, p-CagA, JUN, and fos-like antigen-1 (FOSL1) in AGS and SGC-7901 cells infected with *Helicobacter pylori* (*H. pylori*) for 6 h. $^{a}P < 0.05$; B: RT-qPCR for JUN and dickkopf-related protein 1 (DKK1) in JUN knockdown and/or *H. pylori*-infected AGS and SGC-7901 cells. $^{a}P < 0.05$; $^{b}P < 0.01$; C: Western blotting for JUN, p-JUN, and DKK1 in JUN knockdown and/or *H. pylori*-infected AGS and SGC-7901 cells. $^{a}P < 0.05$; D: RT-qPCR (top) and Western blotting (bottom) for JUN and DKK1 in NCI-N87 and SNU-16 cells with JUN overexpression. $^{a}P < 0.05$; $^{c}P < 0.001$; E: Prediction of AP-1 binding sites in the DKK1 promoter (from -1100 to + 1 bps) by JASPAR; F: JUN and/or FOSL1 promotes DKK1 promoter activity in AGS cells by dual-luciferase reporter assay. The ratio of firefly to Renilla luciferase (Fluc/Rluc) is used to display the results. $^{a}P < 0.01$; $^{c}P < 0.001$; Con: Control; *Hp*: *H. pylori*; GZ7: *H. pylori* GZ7; 26695: *H. pylori* 26695. FOSL1: Fos-like antigen-1; DKK1: Dickkopf-related protein 1; *H. pylori*: *Helicobacter pylori*.

significantly higher in GC tissues than normal tissues, and GC patients with a higher DKK1 expression had a shorter overall and disease-free survival (Figure 3B). Human GC tissues showed stronger positive staining for JUN and DKK1 than pericancer tissues (Figure 3C). However, no distinction was observed in the levels of JUN mRNA between GC and normal tissues in the TCGA-STAD database (Supplementary Figure 2).

Knockdown or overexpression of DKK1 suppresses or promotes malignant behavior of GC cells

To investigate the function of DKK1 in GC, we first established DKK1-knockdown AGS cells using two independent shRNAs (Supplementary Figure 3A) and found that cell proliferation and colony formation were significantly inhibited in AGS cells (Figure 4A and B). DKK1 knockdown also suppressed AGS cell migration and invasion (Figure 4C). Flow cytometry showed that the knockdown of DKK1 greatly increased apoptosis in AGS cells, especially early apoptosis (Figure 4D).

We generated stable DKK1-overexpressing AGS and BGC823 cells (Supplementary Figure 3B). In contrast to DKK1 knockdown, DKK1 overexpression promoted AGS and BGC823 cell growth, colony formation, migration, and invasion (Figure 4E and F). We subcutaneously implanted the DKK1-overexpressing BGC823 cells into nude mice and found that DKK1 overexpression in cancer cells increased the growth of xenograft tumors in mice. The tumor weight and volume in DKK1-overexpressing nude mice were significantly greater than normal control mice (Figure 4G). The mice were killed, and the subcutaneous tumors were removed. Immunohistochemical staining confirmed that DKK1 and Ki67 (cell proliferation marker) were expressed at higher levels in DKK1-overexpressing than control tumors (Figure 4H). These findings verified the tumor-promoting effects of DKK1 in GC.

Binding of DKK1 to CKAP4, but not LRP5/6, in GC tissues and cells

DKK1 suppresses the Wnt/β-catenin pathway by interacting with Wnt coreceptors LRP5/6. Therefore, we detected β-catenin expression and nuclear translocation in DKK1-knockdown AGS cells and discovered that the levels of β-catenin mRNA and protein were reduced by DKK1 knockdown without significant change in β-catenin nuclear translocation, which was even lower in shDKK1-2# cells (Figure 5A-C). In contrast, DKK1 overexpression enhanced β-catenin mRNA levels in AGS and BGC823 cells (Figure 5D). The expression of β-catenin in GC tissues was greater than in normal tissues, but there is no correlation between β-catenin expression and GC patients' survival in the TCGA-STAD dataset (Figure 5E). The findings suggest that the effects of DKK1 are independent of Wnt/β-catenin signaling in GC.

CKAP4 was recently discovered as a DKK1 receptor. We found that CKAP4 mRNA and protein expression was increased in 12 clinical GC samples, especially in the cell surface membrane (red arrow), and 375 GC specimens from the TCGA-STAD database (Figure 5F-H). Infection with *H. pylori* also upregulated CKAP4 expression in GC cells (Figure 5I). Higher CKAP4 expression was linked to lower overall survival in GC patients (Figure 5F). The binding of endogenous DKK1 to CKAP4 was detected in *H. pylori*-infected and uninfected BGC823 cells (Figure 5J).

DKK1 promotes the malignant phenotype of GC cells via the PI3K/AKT/mTOR pathway

After confirming the DKK1/CKAP4 axis in GC cells, we evaluated whether DKK1 knockdown and overexpression affected the PI3K/AKT/mTOR pathway. DKK1 knockdown markedly inhibited the

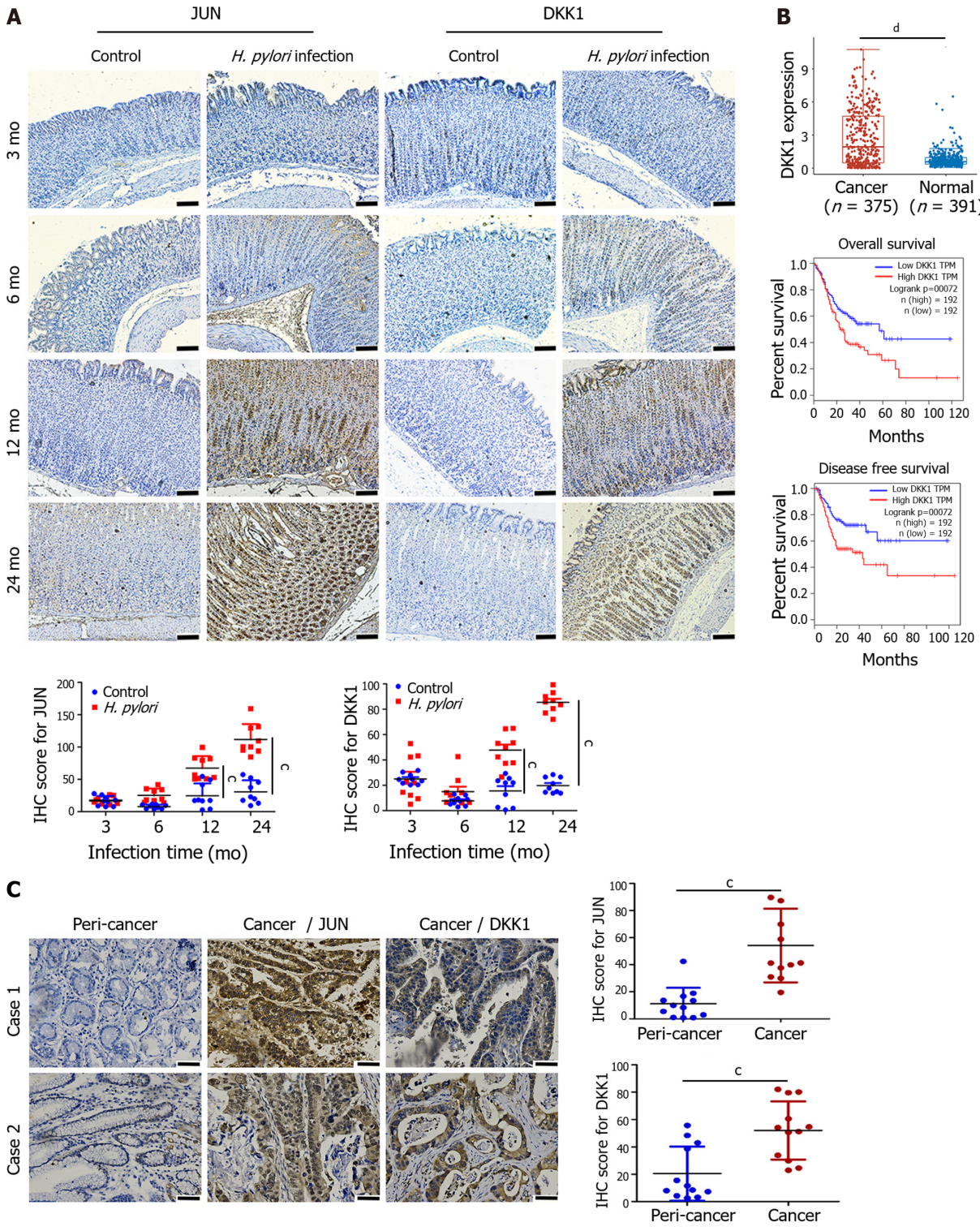

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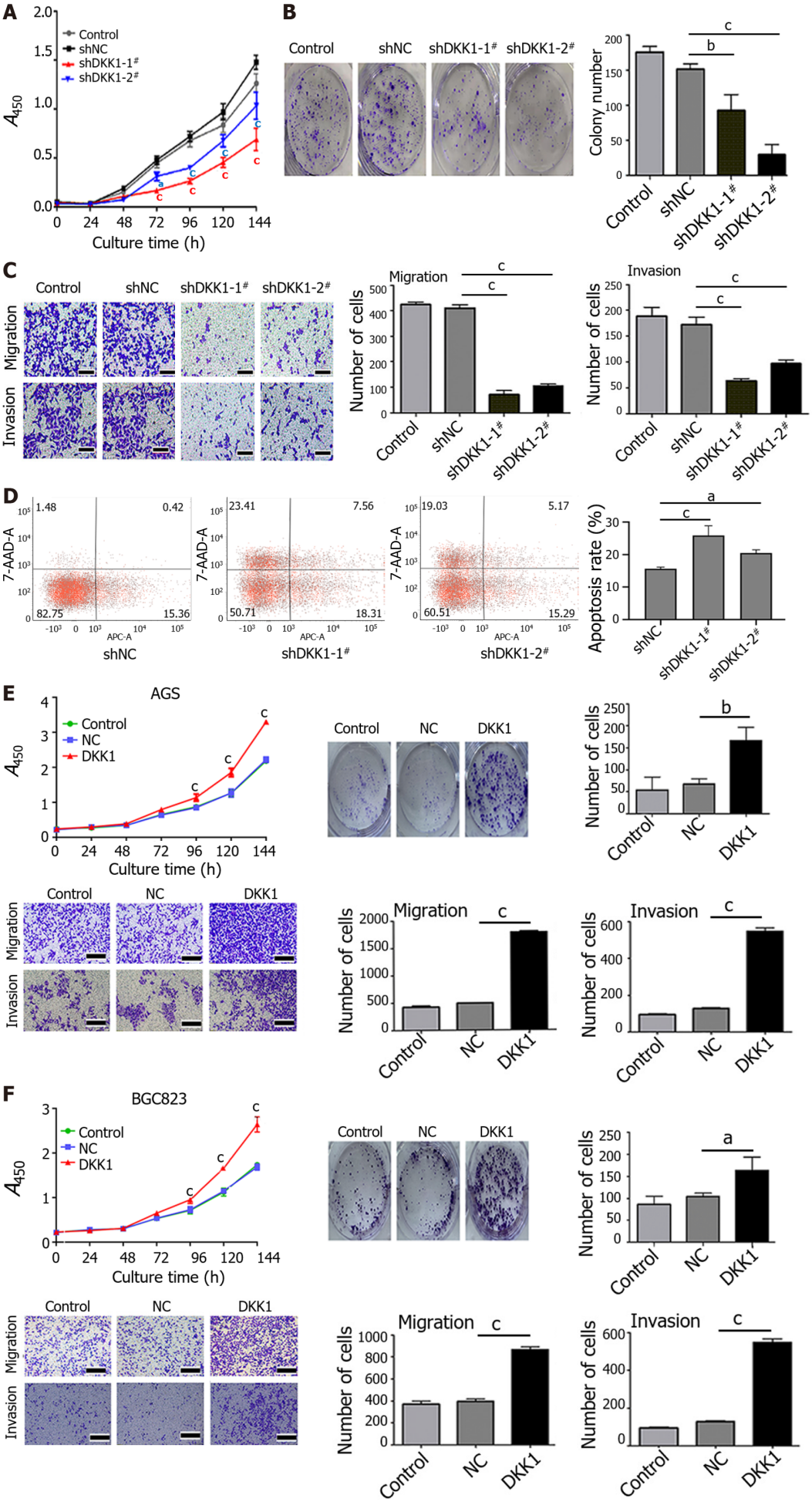
Figure 3 Immunohistochemistry for JUN and dickkopf-related protein 1 in gerbil stomach infected with *Helicobacter pylori* and gastric cancer tissues. A: Immunohistochemical analysis of JUN and dickkopf-related protein 1 (DKK1) proteins in *Helicobacter pylori* (*H. pylori*)-infected gerbil stomach tissues at 3 mo, 6 mo, 12 mo, and 24 mo post-infection (n = 3). Dot diagrams show the quantification of JUN and DKK1 staining in immunohistochemical samples. Nine sections were chosen randomly from three different samples and used to determine mean ± SD of the immunohistochemistry (IHC) score, which was calculated as described in the methods. Scale bar = 100 μm. $^{c}P < 0.001$; B: DKK1 expression and survival analysis in gastric cancer patients from the TCGA-STAD dataset. $^{d}P < 0.0001$; C: Representative images of immunohistochemical staining of JUN and DKK1 proteins in human gastric cancer specimens. Dot diagrams show the quantitation of JUN and DKK1 staining. Scale bar = 50 μm. Data from 12 clinical samples of gastric cancer patients are expressed as mean ± SD. $^{c}P < 0.001$. DKK1: Dickkopf-related protein 1; *H. pylori*: *Helicobacter pylori*.


A
Control
shNC
shDKK1-1#
shDKK1-2#
A450
Culture time (h)
B
Control
shNC
shDKK1-1#
shDKK1-2#
Colony number
C
Migration
Invasion
Number of cells
D
7-AAD-A
APC-A
1.48
0.42
82.75
15.36
23.41
7.56
50.71
18.31
19.03
5.17
60.51
15.29
Apoptosis rate (%)
E
AGS
NC
DKK1
F
BGC823

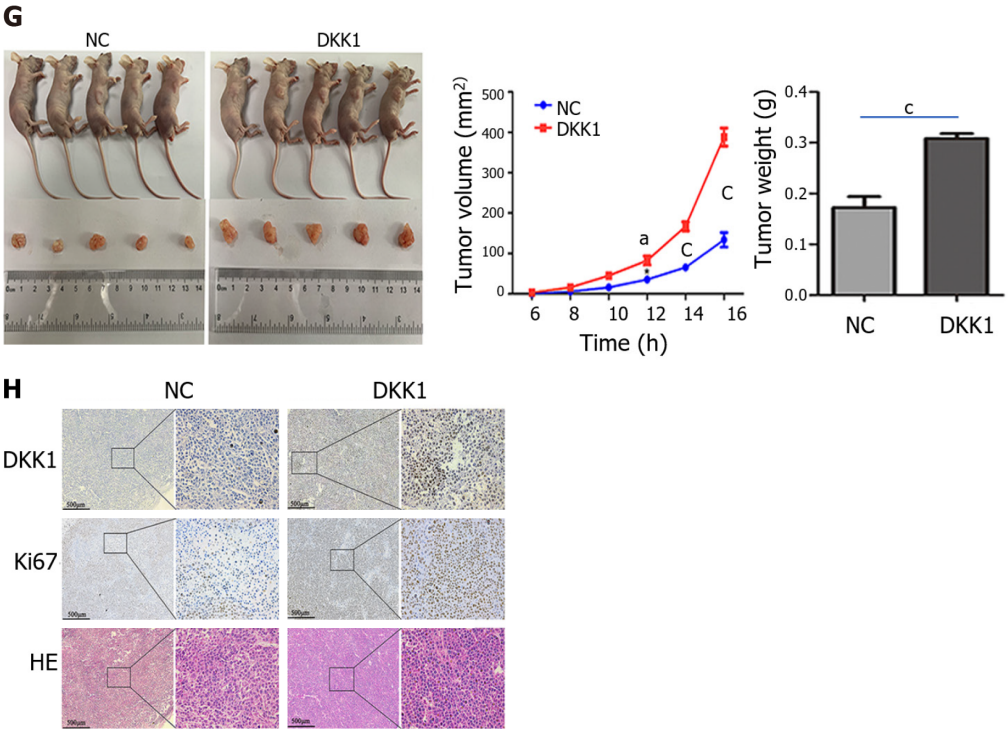


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Figure 4 The effects of dickkopf-related protein 1 knockdown and overexpression on gastric cancer cell growth, migration, invasion, apoptosis, and xenograft growth in nude mice. A: Growth curve of dickkopf-related protein 1 (DKK1) knockdown AGS cells using CCK8 assay. $^{a}P < 0.05$; $^{c}P < 0.001$; B: Colony formation assay of AGS cells with DKK1 knockdown. $^{b}P < 0.01$; $^{c}P < 0.001$; C: Migration and invasion assay of DKK1 knockdown AGS cells using Transwell system. Bar graphs show the number of migrated or invaded cells (right). $^{c}P < 0.001$. Scale bar = 200 μm; D: Flow cytometry analysis for apoptosis in DKK1 knockdown AGS cells. Bar graphs show the percentage of apoptotic cells (right). $^{a}P < 0.05$; $^{c}P < 0.001$; E and F: CCK8, colony formation, and Transwell assays were used to measure the growth curve, colony formation, migration, and invasion of AGS (E) and BGC823 (F) cells with DKK1 overexpression. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$; Scale bar = 200 μm; G: DKK1 overexpression promotes xenograft tumor growth in nude mice. Line dot and bar graph show tumor volume and weight, respectively (n = 5 mice per group). $^{a}P < 0.05$; $^{c}P < 0.001$; H: Hematoxylin eosin staining of xenograft tumors and immunohistochemical analysis of DKK1 and Ki67 in xenograft tumors from nude mice. The boxed regions are magnified to the right. Scale bar = 500 μm. DKK1: Dickkopf-related protein 1; HE: Hematoxylin eosin.

expression of p-PI3K, PI3K, p-AKT, AKT, p-mTOR, and mTOR, and p-PI3K, p-AKT, and p-mTOR were more inhibited. *H. pylori* infection restored the expression of DKK1 and increased PI3K/AKT/mTOR signaling in DKK1 knockdown cells (Figure 6A). Conversely, DKK1 overexpression increased PI3K, AKT and mTOR phosphorylation, and a specific inhibitor of PI3K, LY294002, inhibited the DKK1-induced increase in the three phosphorylated proteins in AGS and BGC823 cells (Figure 6B). These findings suggest that *H. pylori*-upregulated DKK1 activated the PI3K/AKT/mTOR pathway.

Next, we examined the effects of LY294002 in DKK1-overexpressing AGS and BGC823 cells and found that blockade of PI3K/AKT/mTOR signaling with LY294002 significantly decreased cell proliferation, colony formation, migration and invasion (Figure 6C and D), which further confirmed our hypothesis that DKK1 promoted the malignant phenotypes of GC cells *via* activation of the PI3K/AKT/mTOR pathway.

DISCUSSION

Our current study revealed that *H. pylori* infection upregulated the expression of JUN and FOSL1, which formed AP-1 to activate DKK1 transcription. Gain- and loss-of-function studies showed that DKK1 had important tumor-promoting functions in *H. pylori*-related GC *via* activation of the CKAP4/PI3K/AKT/mTOR pathway.

The gene expression profiles obtained by independent research groups using RNA-seq were inconsistent due to the use of diverse cell lines, GC tissues, *H. pylori* strains, and whole-genome expression arrays[31-33]. The upregulated expression of JUN and FOLS1 was noted in *H. pylori* NCTC11639-infected AGS cells, but not experimentally verified[34]. DKK1 was also downregulated in *H. pylori* 26695-infected AGS cells, but this observation was not tested experimentally[35]. Using RNA-seq, we identified 32 DEGs that were shared by all three models of primary GC cells infected with *H. pylori* GZ7. Of the 32 DEGs, JUN, FOSL1 and DKK1 were highly expressed in *H. pylori*-infected cells, which was confirmed in GC tissues and *H. pylori*-infected gerbil stomach tissues. The enrichment

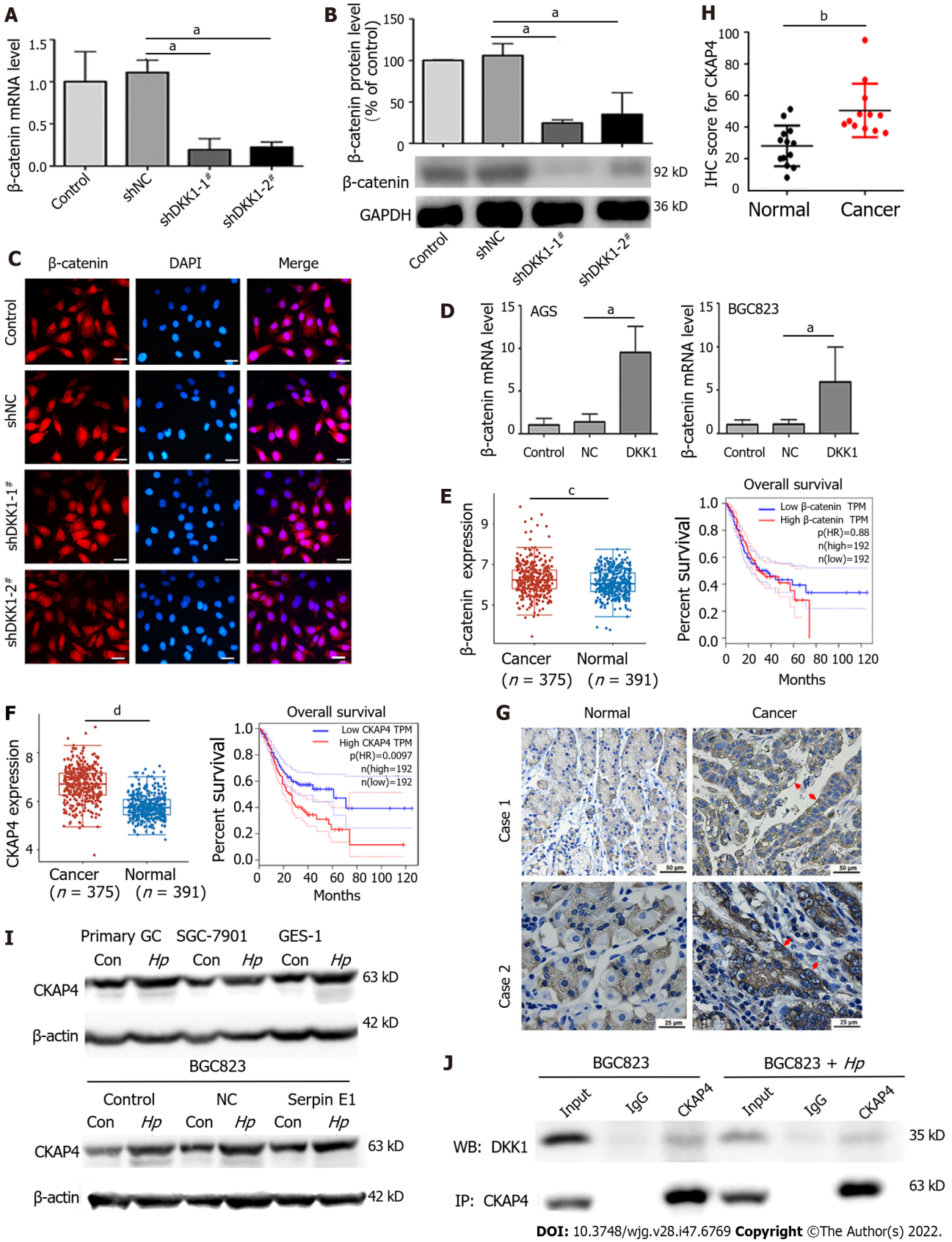

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Figure 5 Dickkopf-related protein 1 promotes β-catenin and cytoskeleton-associated protein 4 expression and the binding of dickkopf-related protein 1 to cytoskeleton-associated protein 4 in gastric cancer tissues and cells but does not affect the nuclear translocation of β-catenin. A-C: Dickkopf-related protein 1 (DKK1) knockdown decreases β-catenin expression without changing β-catenin nuclear translocation in AGS cells, as determined using RT-qPCR (A), Western blotting (B), and immunofluorescence (C). Scale bar = 25 μm; $^{a}P < 0.05$; D: RT-qPCR for β-catenin in AGS and BGC823 cells with DKK1 overexpression. $^{a}P < 0.05$; E: β-catenin expression and its association with 10-year overall survival in gastric cancer (GC) samples of the TCGA-STAD database. $^{c}P < 0.001$; F: Cytoskeleton-associated protein 4 (CKAP4) expression and its positive association with 10-year overall survival in GC samples of the

TCGA-STAD database. [d]*P* < 0.0001; G: Immunohistochemical staining of CKAP4 in clinical GC tissues and matched normal tissues. The red arrow indicates CKAP4 expression in the cell membrane; H: CKAP4 staining quantification in G. Data from 12 independent samples are expressed as means ± SD. [b]*P* < 0.01; I: Western blotting for CKAP4 in GC cells with or without *Helicobacter pylori* (*H. pylori*) infection for 72 h; J: Co-IP of DKK1 and CKAP4 in BGC823 cells with and without *H. pylori* infection for 72 h. Con: Control; *Hp*: *H. pylori*. DKK1: Dickkopf-related protein 1; *H. pylori*: *Helicobacter pylori*; CKAP4: Cytoskeleton-associated protein 4; GC: Gastric cancer.

analysis of KEGG pathways suggested that DEGs were primarily involved in inflammatory signalings such as TNF, NF-κB and IL-17. GESA also revealed that *H. pylori* infection activates the TNFA pathway, the P53 pathway, apoptosis, hypoxia, and the inflammatory response.

DKK1 was recently found to be epigenetically downregulated by promoter hypermethylation, which resulted in the nuclear translocation of β-catenin and activation of the Wnt/β-catenin pathway in gastric intestinal metaplasia, high-grade adenoma, and adenocarcinoma[36,37]. Conversely, Kikuchi *et al*[38] observed that DKK1 promoted the proliferation, invasion and metastasis of cancer cells independent of Wnt signaling. Other studies also suggested that DKK1 was often overexpressed in diverse tumor tissues, including GC, and DKK1 overexpression strongly correlated with poor cancer patient survival [39,40]. However, the mechanism underlying this association was not clear. Our study explained, for the first time, why DKK1 was upregulated in GC. *H. pylori* infection upregulates JUN and FOSL1 expression to form AP-1. AP-1 binds to the TRE and CRE motifs in the DKK1 promoter region to transcriptionally activate DKK1 expression in GC. This finding is contradictory to two earlier studies[36,37]. We speculate that decreased expression of DKK1 *via* promotor methylation, as reported by Lu *et al*[36], is more prevalent in gastric precancerous lesions and early-stage adenocarcinoma, and increased expression of DKK1 *via* promotor binding of AP-1, as observed in our study, is more likely to occur in *H. pylori*-infected and advanced GC. Two observations provide support for our assertion. First, we discovered that high expression of JUN and DKK1 was mostly observed in the stomach tissues of gerbils at least 12 mo after *H. pylori* infection. Second, DKK1 is frequently overexpressed in GC patients with lymph node invasion and distant metastasis[41].

We demonstrated that the cancer-promoting function of DKK1 in GC was independent of Wnt/β-catenin signaling by observing that β-catenin expression and nuclear translocation were inhibited in DKK1-knockdown cells. CKAP4 was initially identified as an endoplasmic reticulum protein that was trafficked to the cell surface membrane after palmitoylation[42]. The combination of CKAP4 and DKK1 on the cell surface triggers pro-oncogenic or tumor-suppressive DKK1 signaling in different tumors[43, 44]. Although CKAP4 expression is high in many tumors[45,46], there is no information on CKAP4 expression in GC. We discovered that *H. pylori* infection simultaneously increased the expression of DKK1 and CKAP4 in GC cells, particularly CKAP4 expression on the cell membrane. High levels of DKK1 and CKAP4 expression were also consistently observed in GC tissues from the TCGA-STAD database and clinical GC specimens and were linked to a poor prognosis for GC patients. Notably, the interaction between DKK1 and CKAP4 was detected in GC cells. These results show that the DKK1/CKAP4 axis is present in *H. pylori*-infected GC cells and GC tissues.

There are two tandem cysteine-rich domains in the DKK1 promoter region, CRD1 and CRD2. DKK1 CRD1 domain binding to LPR6 inhibits Wnt signaling, and DKK1 CRD2 domain binding to CKAP4 activates PI3K/AKT signaling[38]. CKAP4 overexpression on the surface of cancer cells likely prevents DKK1 binding to LRP6, resulting in subsequent suppression of Wnt/β-catenin signaling[47]. This presumption is partially supported by our experimental results that *H. pylori*-induced DKK1 activated the CKAP4/PI3K/AKT/mTOR pathway rather than inhibiting LRP6/Wnt/β-catenin signaling in GC cells. Infection with *H. pylori* reversed the PI3K/AKT/mTOR signaling inhibition caused by DKK1 knockdown. PI3K inhibition with LY294002 also suppressed the DKK1-mediated activation of the PI3K pathway and the malignant phenotype of GC cells. Recent evidence has shown that the PI3K/AKT/mTOR and Wnt/β-catenin signalings are commonly activated in GC[48,49]. However, the reason for DKK1 selective activation of the CKAP4/PI3K/AKT/mTOR pathway in *H. pylori*-related GC is not known and requires further study.

There were several limitations to this study. First, the number of clinical samples was small. Therefore, further study with larger sample sizes is required to determine the expression of the DKK1/CAKP4 axis in GC tissues and its association with *H. pylori* infection in cancer tissues. Second, only two strains of *H. pylori* were used in this study: An East Asian strain (*H. pylori* GZ7) and a western strain (*H. pylori* 26695). However, *H. pylori* exhibit intrastrain and interstrain heterogeneity. More *H. pylori* strains will be required to verify our findings.

CONCLUSION

H. pylori infection upregulated JUN and FOSL1 expression, which formed AP-1 to promote DKK1 transcription that resulted in gastric tumorigenesis *via* activation of the CKAP4/PI3K/AKT/mTOR pathway. DKK1/CKAP4 interaction could become an attractive target for *H. pylori*-related GC therapy.

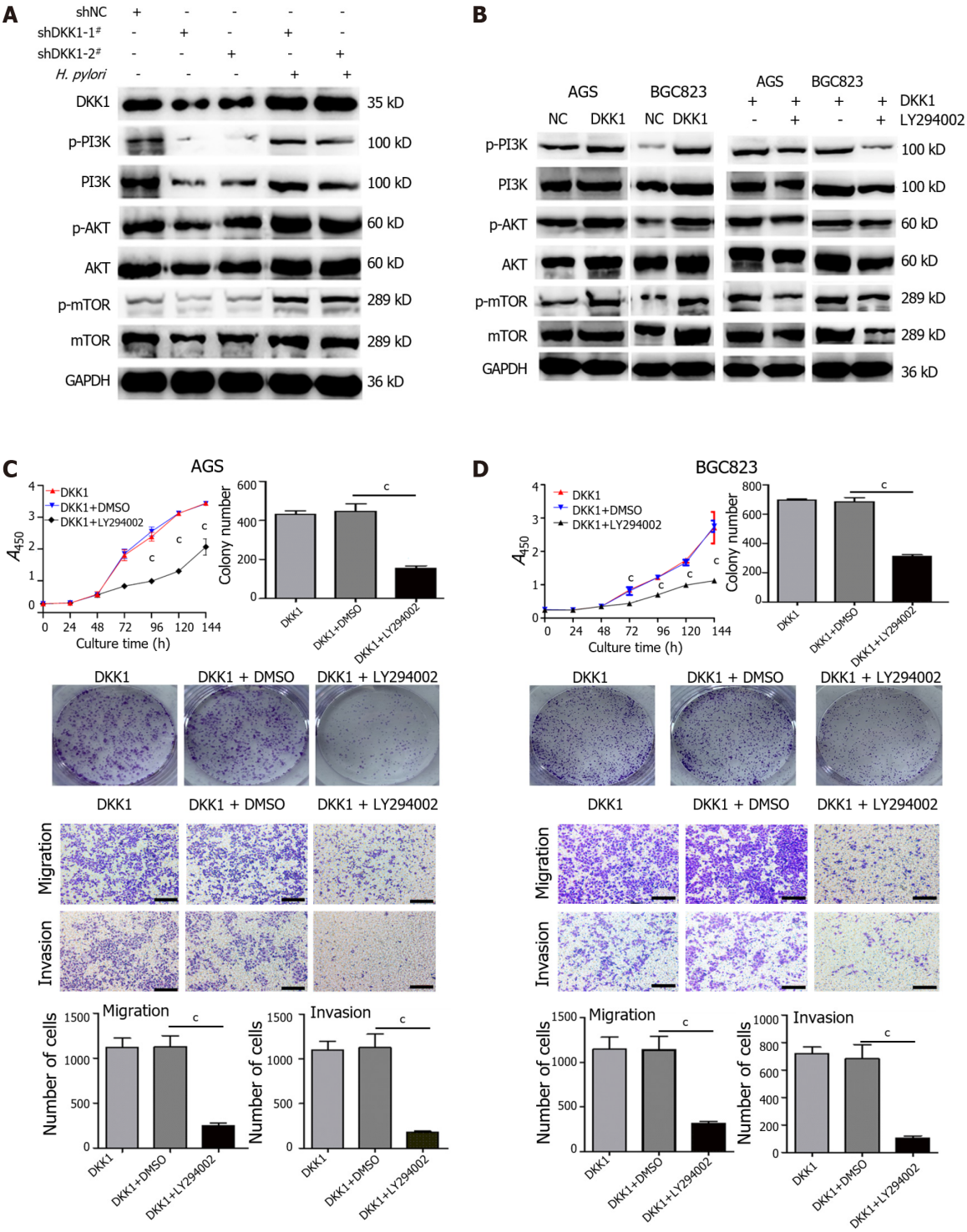

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Figure 6 Dickkopf-related protein 1 promotes the growth, migration, and invasion of AGS and BGC823 cells by activating the phosphatidylinositol 3-kinase/AKT/ mammalian target of rapamycin pathway. A and B: Western blotting for phosphatidylinositol 3-kinase (PI3K), p-PI3K, AKT, p-AKT, mammalian target of rapamycin (mTOR), and p-mTOR in dickkopf-related protein 1 (DKK1) knockdown and/or *Helicobacter pylori*-infected AGS cells (A) and DKK1 overexpression and/or LY294002 (50 μmol/L)-treated AGS and BGC823 cells (B); C and D: LY294002 treatment (50 μmol/L) decreases cell proliferation, colony formation, migration, and invasion in DKK1 overexpression AGS (C) and BGC823 (D) cells. Bar graphs show the quantitation of colony numbers or migrated and invaded cell numbers. Scale bar = 200 μm. $^{c}P < 0.001$. DKK1: Dickkopf-related protein 1; *H. pylori*: *Helicobacter pylori*; PI3K: Phosphatidylinositol 3-kinase; mTOR: Mammalian target of rapamycin.

The identification of small compounds and drugs targeting the DKK1/CKAP4 axis will be a crucial aspect of future studies. We will also investigate this possibility further.

ARTICLE HIGHLIGHTS

Research background

Gastric cancer (GC) is one of the most common malignant tumors with a high morbidity and mortality rate globally, especially in East Asian countries. *Helicobacter pylori* (*H. pylori*) infection is the most significant risk factor for GC. Studying their interaction can reveal the potential pathogenesis and therapeutic targets of GC.

Research motivation

Although substantial efforts have been done to link *H. pylori* infection and GC over the past decades, the molecular mechanisms of *H. pylori*-induced GC are not fully understood, which results in reduced treatment benefits.

Research objectives

The present study aimed to study the interaction of *H. pylori* infection, dickkopf-related protein (DKK) 1, and cytoskeleton-associated protein (CAKP) 4 in GC and the underlying molecular mechanisms.

Research methods

RNA sequencing identified differentially expressed genes (DEGs) between *H. pylori*-infected and uninfected primary GC cells. Dual-luciferase reporter assay and co-immunoprecipitation determined the interaction of activator protein (AP)-1, DKK1 and CKAP4. Western blotting and immunohistochemistry detected the expression of DKK1, CKAP4 and phosphatidylinositol 3-kinase (PI3K) pathway-related proteins in GC cells and tissues. Functional experiments and tumorigenicity in nude mice detected the malignant behavior of GC cells *in vitro* and *in vivo*.

Research results

H. pylori infection upregulated JUN, FOSL1, DKK1 and CKAP4 expression in GC cells, *H. pylori*-infected gerbil gastric tissues, and human GC samples. JUN and FOSL1 formed activator protein-1 (AP-1) to transcriptionally activate DKK1. DKK1 bound to CKAP4, but not Wnt coreceptor, to promote GC cell growth, migration, invasion, and xenograft tumor growth in nude mice *via* activating the PI-3K/AKT/mammalian target of rapamycin (mTOR) pathway. Targeting PI3K inhibited DKK1-mediated CKAP4/PI3K signaling activity and the malignant behavior of GC cells.

Research conclusions

H. pylori-induced AP-1 promotes the binding of DKK1 to CAKP4, which contributes to gastric tumorigenesis *via* the PI3K/AKT/mTOR pathway.

Research perspectives

The findings suggest that the DKK1/CKAP4 interaction may be a therapeutic target for *H. pylori*-induced GC. The identification of small compounds and drugs targeting the DKK1/CKAP4 axis will be a crucial aspect of future studies. We will also investigate this possibility further.

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FOOTNOTES

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LETTER TO THE EDITOR

The potential role of the three-dimensional-bioprinting model in screening and developing drugs

Chao-Lin Deng, Bin Wu

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Abstract

Recently, we have read with great interest the original article used different spatial configuration models of colorectal cancer (CRC) for validating the anti-tumor efficacy with Diiminoquinone. We feel obliged to provide new insight into the drug screening models by integrating and analyzing the original method and result. These comments may provide comprehensive insights into three-dimensional drug screening models and the difference between pathologic subtypes in CRC.

Key Words: Colorectal cancer; three-dimensional-bioprinting; Mucinous adenocarcinoma; Drug screening models

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Core Tip: Chemotherapy is the main treatment option for inoperable colorectal cancer (CRC). We recently read an article about the anti-cancer effects of Diiminoquinone. We feel obliged to express our opinion on this article on drug screening models and the difference between pathologic subtypes in CRC and hope it could deepen understanding for the reader.

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TO THE EDITOR

We have read with great interest the article by Monzer *et al*[1]. The authors present a novel drug, Diiminoquinone (DIQ), with inhibitory effects for colorectal cancer (CRC) in different spatial configuration models. In vivo tests, Similar results have been obtained for drug effectiveness. In conclusion, the authors showed that DIQ may through suppresses Wnt/-catenin, AKT, and ERK pathways to the tumor and thereby inhibits tumor progression with significant potential to be translated into clinical practice.

The highlight of this study is that the authors used multiple three-dimensional (3D) models to verify the effectiveness of DIQ. The two-dimensional (2D) monolayer model has long been used *in vitro* cancer research for novel drug development and screening. However, 2D cancer cell models dramatically differ from cancer in vivo. Without spatial configurations, oncometabolite around the tumor microenvironment (TME)[2,3], and intercellular signaling between the cancer cell and other cells, the result from the 2D module may be unable to draw correct conclusions, and this causes further challenges for clinic translation. In this research, sphere formation assays with tumor cell lines and derived organoids were established and used to prove the safety and efficacy of DIQ and to reflect more accurately drug sensitivity measurements result.

We found some details through in-depth analysis and hope to express some relevant views. 3D culture models should ideally recapitulate the native TME. Despite sphere formation as a classic approach for 3D models, the limitation of this method is the lack of intercellular communication in multiple cell types. However, 3D-bioprinting provides several critical advantages over sphere formation assay in drug development or screening, such as using bio-ink to simulate the cytoskeleton or partial tumor tissue with multi-cell to a highly complex hierarchical 3D structure. These configurational were able to enhance intercellular communication and signaling factors transportation and provide a more accurate result for novel drug development[4,5]. Although the authors used organoid cultures to verify the drug's effectiveness at a later stage, the success rate of organ-like laboratory cultures is too low, which significantly limits the possibility of large-scale experimental validation. If 3D bioprinting is used, the required tissue size and culture conditions are lower than those of organoid cultures, which seems to provide more experimental samples for drug validation and enhance the data grade of this drug for clinical validation. Various 3D-bioprinting models were established, which aimed at disease modeling, novel drug development, and biological function evaluation[3,6]. Therefore, based on the current research data, tumor modeling using 3D bioprinting technology after primary cell cultures seems to be more beneficial for chemotherapy drug sensitivity screening.

Another interesting finding was that the DIQ showed chemotherapy effectivity in mucinous adenocarcinoma (MC), a unique pathological subtype of CRC[7]. In a previous study, the chemosensitivity of MC was poor either irinotecan- or oxaliplatin-based therapeutic strategies than in non-mucinous tumors[8]. One MC patient tissue was successfully grown as an organoid model in the paper, which does not seem to provide sufficient evidence for the effectiveness of DIQ for colorectal MC. Nevertheless, the authors' experimental results provide a possible research direction for chemotherapy targeting pathological subtypes.

This original article uses multiple models of CRC to demonstrate DQI as a potential novel drug for chemotherapy. However, further research is needed to support the safety and efficacy of clinical translation.

FOOTNOTES

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