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Contents

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EDITORIAL

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EDITORIAL

Role and timing of endoscopy in acute biliary pancreatitis

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Abstract

The role and timing of endoscopy in the setting of acute biliary pancreatitis (ABP) is still being debated. Despite numerous randomized trials have been published, there is an obvious lack of consensus on the indications and timing of endoscopic retrograde cholangiopancreatography (ERCP) in ABP in meta-analyses and nationwide guidelines. The present editorial has been written to clarify the role of endoscopy in ABP. In clinical practice the decision to perform an ERCP is often based on biochemical and radiological criteria despite they already have been shown to be unreliable predictors of common bile duct stone presence. Endoscopic ultrasonography (EUS) is not currently a worldwide standard diagnostic procedure early in the course of acute biliary pancreatitis, but it has been shown to be accurate, safe and cost effective in diagnosing biliary obstructions compared with magnetic resonance cholangiopancreatography and ERCP and therefore in preventing unnecessary ERCP and its related complications. Early EUS in ABP allows, if appropriate, immediate endoscopic treatment and significant spare of unnecessary operative procedures thus reducing possible related complications.

Key words: Acute biliary pancreatitis; Choledocolithiasis; Common bile duct stone; Endoscopic retrograde cholangiography; Endoscopic ultrasonography; Endoscopic ultrasonography

Core tip: Although several reports have been published on role and timing of endoscopy in the treatment of acute biliary pancreatitis (ABP), there are still some controversial in this subject. In clinical practice the decision to perform an endoscopic retrograde cholangiopancreatography is often based on biochemical and radiological criteria despite they already have been shown to be unreliable predictors of common bile duct (CBD) stone presence. Both magnetic resonance cholangiopancreatography and endoscopic ultrasonography (EUS) are now indicated as the best noninvasive imaging methods for CBD stone detection. Early EUS in ABP allows, if appropriate, immediate endoscopic treatment and significant spare of unnecessary operative procedures thus reducing possible related complications.

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The role and timing of endoscopy in the setting of acute biliary pancreatitis (ABP) is still being debated. A recent systematic review by van Geenen *et al*[1] clearly demonstrated that, despite numerous randomized trials, there is an obvious lack of consensus on the indications and timing of endoscopic retrograde cholangiopancreatography (ERCP) in ABP in meta-analyses and nationwide guidelines. Although the indication of early (within 24-48 h) ERCP with papillosphincterotomy for patients with ABP and related cholangitis is well established[2,3], its role in cases of either mild or severe ABP, without signs of cholangitis, remains controversial.

Biliary pancreatitis results from the migration of a gallstone to the common bile duct (CBD) with impaction or temporary obstruction of the major duodenal papilla[2]. Most ABP attacks are not severe, are self-limiting, and improve with conservative management[4]. Spontaneous passage of CBD stones in the duodenum has been described in up to 50% of cases of ABP[5,6]. However, conservative management of these patients is associated with a biliary complication rate of up to 20%. In such cases, ERCP is delayed and may be performed under possibly more difficult conditions, thus increasing the failure rate[7,8]. Moreover, without definitive treatment, the risk of a recurrent attack within the next several months is about 30%-50%[9,10]. Even after a mild attack, cholecystectomy and/or biliary sphincterotomy should be considered within weeks[11]. In a large retrospective study, Nguyen *et al*[12] demonstrated that hospital readmission rates for ABP within 12 mo were significantly reduced with cholecystectomy (14.0% *vs* 5.6%) or ERCP (13.1% *vs* 5.1%).

In clinical practice, the decision to perform early ERCP is often based on biochemical and radiological criteria, such as the presence of cholestatic liver biochemistry and a dilated CBD. Nevertheless, studies have shown that commonly used biochemical and radiological predictors of the presence of CBD stones in patients with ABP are unreliable[13]. Even with the application of various clinical predictors, only 37%-42% of patients undergoing ERCP were found to have CBD stones[14,15].

The rate of complications after therapeutic ERCP ranges from 7% to 10% and the mortality rate from 0.2% to 2.2%[16,17]. Therefore, accurate prediction of CBD stones is warranted to select patients for early therapeutic ERCP. Other noninvasive (or minimally invasive) imaging techniques such as endoscopic ultrasonography (EUS) and magnetic resonance cholangiopancreatography (MRCP) have been used to select patients for therapeutic ERCP to minimize the risk of complications associated with unnecessary diagnostic ERCPs. Both EUS and MRCP have been confirmed in meta-analyses to be highly accurate for the diagnosis of CBD stones[18,19], with similar sensibility, specificity, accuracy, negative predictive value, and positive predictive value for detection of CBD stone[20].

In case of ABP without signs of cholangitis, the American guidelines[21] suggest performing EUS or MRCP prior to ERCP depending on the local expertise and facilities. Although MRCP also provides excellent imaging of the biliary tree, EUS is more accurate in the detection of small stones (< 5 mm), which are responsible for at least half of all cases of acute pancreatitis, and is better for visualizing microlithiasis of the gallbladder[21]. Indeed, despite the fact that most stones pass spontaneously, establishing a biliary etiology is extremely important because there is a high risk of recurrent pancreatitis (33%-60%) if the gallstone disease is not treated[22-24].

The relative sensitivity of MRCP and EUS for the detection of CBD stones use as a reference standard the extraction of CBD stones after endoscopic sphincterotomy during ERCP[25]. However, it is well known that small stones can be missed even during therapeutic ERCP. Therefore, EUS has recently been proposed as the new gold standard in the diagnosis of choledocholithiasis[26].

In 2001, Scheiman *et al*[27] prospectively compared the clinical efficacies of EUS and MRCP when performed within 24 h before ERCP in patients with biliary disease. They reported that although MRCP had the lowest procedural reimbursement, the initial EUS strategy had the greatest cost utility by avoiding unnecessary ERCP examinations. Thus, the selection of endoscopic treatment based on EUS may eventually impact the treatment of ABP and provide greater safety for the patients, as well as more rational use of healthcare resources[28]. A preliminary EUS may help in decision-making: if a stone is present, ERCP with extraction can be performed in the same endoscopic session, whereas if no stone is found, the patient can be spared the added risk. This stepwise strategy has been shown to help avoid unnecessary ERCP in most patients[29].

Certainly, either EUS or MRCP can be chosen based on local availability[30]. Postponing treatment for symptomatic CBD stones exposes the patient to biliary complication, especially cholangitis[31]. Moreover, in a 2008 editorial on gastrointestinal endoscopy, Savides noted that even if MRCP reveals a CBD stone, it is still worth considering an EUS immediately before the ERCP because approximately 21% of CBD stones (especially those < 8 mm) can pass spontaneously, which could occur in the interval between MRCP and ERCP[5,32]. In many centers and in real-life practice, timing and availability of MRCP precludes its

acceptability as a method for determining the need for prompt ERCP, whereas EUS is more readily accessible.

EUS is not currently a worldwide standard diagnostic procedure early in the course of ABP, but because of its accuracy, safety, and cost effectiveness in diagnosing biliary obstructions compared with MRCP and ERCP, we think it should be considered as the first choice in approaching ABP. EUS is also a preferable diagnostic choice because it can be performed at the bed side of the patient, which is especially relevant for patients in an ICU. An early (within 24-48 h) EUS can easily and quickly categorize those patients who do not require subsequent therapeutic ERCP, thus allowing even an early discharge in select cases, which is important in terms of cost effectiveness.

EUS and MRCP are now considered alternative noninvasive methods for evaluating biliary obstruction, and guidelines suggest performing one or the other prior to therapeutic ERCP depending on local availability. However, we think it is important to have a more rational use of healthcare resources while trying to follow the best clinical practice, rather than mainly adapting our practice to the resources available locally. Ideally, we should aim to have an integrated gastroenterology unit that can manage CBD stones by a combined, simultaneous two-step approach, and gastroenterologists responsible for ERCP should be trained in EUS and *vice versa*[33].

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TOPIC HIGHLIGHT

2015 Advances in Gastrointestinal Endoscopy

How to establish endoscopic submucosal dissection in Western countries

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Abstract

Endoscopic submucosal dissection (ESD) has been invented in Japan to provide resection for cure of early cancer in the gastrointestinal tract. Professional level of ESD requires excellent staging of early neoplasias with image enhanced endoscopy (IEE) to make correct indications for ESD, and high skills in endoscopic electrosurgical dissection. In Japan, endodiagnostic and endosurgical excellence spread through personal tutoring of skilled endoscopists by the inventors and experts in IEE and ESD. To translocate this expertise to other continents must overcome two fundamental obstacles: (1) inadequate expectations as to the complexity of IEE and ESD; and (2) lack of suitable lesions and master-mentors for ESD trainees. Leading endoscopic mucosal resection-proficient endoscopists must pioneer themselves through the long learning curve to proficient ESD experts. Major referral centers for ESD must arise in Western countries on comparable professional level as in Japan. In the second stage, the upcoming Western experts must commit themselves to teach skilled endoscopists from other referral centers, in order to spread ESD in Western countries. Respect for patients with early gastrointestinal cancer asks for best efforts to learn endoscopic categorization of early neoplasias and skills for ESD based on sustained cooperation with the masters in Japan. The strategy is discussed here.

Key words: Endoscopic submucosal dissection; Early cancer; Endoscopic submucosal dissection clinical tutoring; Endoscopic submucosal dissection training; Gastrointestinal neoplasias; Endoscopic submucosal dissection learning curve; Endoscopic submucosal dissection techniques; Endoscopic submucosal dissection complications

Core tip: Endoscopic submucosal dissection (ESD) was developed in Japan for curative resection of early cancer. But Western countries take very long without tutoring to establish ESD on a professional level. A two-fold, sequential learning curve is necessary for endoscopic staging, and for endoluminal surgery of early neoplasias. This will need a sequential strategy: (1) education for diagnostic skills in routine endoscopy and in educational programs; and (2) endoscopists proficient in endoscopic snaring techniques must train for ESD and pass an untutored learning curve to become proficient. Then, Western ESD experts must instruct endoscopists from referral centers in their country.

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INTRODUCTION

Endoscopic submucosal dissection (ESD) using electroknives was developed in Japan for curative *en-bloc* resection of early gastric cancer without risk of lymph node metastasis (LNM)[1,2]. Cancer of the gastrointestinal (GI) tract only is curable by resection. The obvious advantage of ESD over endoscopic mucosal resection (EMR) by electrosnaring is the ability to achieve *en-bloc* resection of even extended early neoplasias yielding accurate histological diagnosis and minimal recurrence rate[3]. EMR of larger lesions (> 2 cm) results in few or multiple pieces (piecemeal, PM), indeterminate histological resection status and high recurrence rate[3]. In contrast to surgery, ESD leaves the GI tract intact preserving the patient´s quality of life[4-6].

The decision for ESD or surgical full-wall resection with lymphadenectomy - is made by endoscopic staging of superficial gastrointestinal neoplasias using image-enhanced endoscopy (IEE, magnifying chromo- or NBI-endoscopy)[6,7]. Therefore, endoscopic findings describe the classical and expanded indication criteria for curative ESD (intention-to-treat)[4,8-16] (Table 1) that aim for curative resection by histologic outcome[17] (Table 2). Before resection, the neoplasia is usually confirmed by just a single targeted biopsy. *Classical* and *Expanded Indications* in *Stomach* and *Esophagus*, and *Indications* in *Colorectum* have been defined[1,8,10-14,16], evaluated by curative resection rates > 80%[18-23], and confirmed by excellent recurrence-free 3- to 5-years survival rates (> 96%) in large ESD studies performed by proficient operators in Japan[18,23-30]. ESD has replaced EMR throughout Japan as state-of-the-art therapy for a wide spectrum of pre-/malignant early neoplasias in stomach, esophagus and colorectum[3].

ESD technique has rapidly spread throughout Japan, because EMR-experienced endoscopists acquired the skills in clinical procedures under supervision by ESD experts and had a high case load of gastric neoplasias most suitable for learners[31]. The goal of the early learning curve is to achieve competence level, as defined by *en-bloc* resections in > 80% and complications in less than 10% of ESD procedures[31], qualifying to perform untutored ESD procedures. A skilled and well prepared endoscopist usually attains competence for gastric ESD after approximately 30 tutored procedures[31-34], and then proceeds with 30 to 40 tutored procedures for competence in colorectal ESD[35-38]. Even without experience in gastric ESD, about 40 tutored colorectal procedures are sufficient to attain competence level for colorectal ESD[35-37].

The ESD technique is quite slowly transferred to Western countries, because they must acquire double expertise - diagnostic and electrosurgical - and early gastric neoplasias most suitable for learning ESD are too rare[31,39]. A systematic strategy (Figure 1) to establish proficient ESD in Western countries needs to build on Western experience the main topics - Preparations for and Training in ESD, Clinical Learning Curve in ESD, and Continued Medical Education in IEE and ESD.

WESTERN EXPERIENCE IN ESD

Over the past six years smaller prospective series were published from pioneering centers in Western countries. There were few preliminary reports without long-term outcome on heterogenous initial series from single centers[40-43] or cumulative multiinstitutional registries[44,45]. The prospective series with follow-up on gastric[46-51], esophageal[52-57], and colorectal ESD[38,58-61] all focussed on rate of *en-bloc* resection [median 92% (range 68%-100%)], complications [median 13% (7%-27%)] and speed of dissection. These series reported only moderately lower rates of *en-bloc* resection [median 92% (68%-100%)] and recurrence-free survival [median 96.7% (91%-100%)] at 1-2 years than in Japan. However, rates of curative resection were inferior, median 72%-75% per organ, but lowest rates per study were 64% for gastric cancer, 46% for esophageal squamous cell cancer, 39% for early Barrett adenocarcinoma, and 7%

Table 1 Indications for endoscopic *en-bloc* resection of gastrointestinal neoplasias (modified from[6])

Organ	Indications for ...	Ref.
Stomach	ESD - classical indications[1] mucosal adenocarcinoma; intestinal type G1 or G2, size d ≤ 2 cm, no ulcer ESD - expanded indications[2] adenocarcinoma, intestinal type, G1 or G2, any size without ulcer/adenocarcinoma, intestinal type, G1 or G2, sm-invasive < 500 μm/adenocarcinoma, intestinal type, G1 or G2, d ≤ 3 cm, with ulcer/adenocarcinoma diffuse type, G3 or G4, size d ≤ 2 cm, no ulcer	[1,4,5,13]
Esophagus	ESD - classical indications[1] SCC type 0-IIb (HGIN or G1, G2), intramucosal (m1, m2), any size Barrett adenoca. type 0-II (G1, G2), intramucosal (m1, LPM), no ulcer ESD - expanded indications[2] SCC type 0-II (HGIN, G1, G2) slightly invasive (m3, sm < 200 μm), any size[3], clinical N 0 Barrett adenocarcinoma type 0-II (HGIN or G1, G2), mucosal (≤ MM), clinical N 0	[5,8,9,12,14,15]
Colorectum	ESD Indications Any neoplasias > 20 mm in diameter without signs of deep submucosal invasion, indicative for *en-bloc* resection and unsuitable for EMR *en-bloc*: LST-granular type d ≥ 4 cm (villous adenoma +/- HGIN)[4] LST-nongranular type d ≥ 2 cm Mucosal carcinoma (HGIN, G1 or G2), or superficially sm-invasive[5] Depressed-type neoplasias (0-IIc) Neoplasias type 0-I or 0-II with pit pattern type VI (irregular) Sporadic localized neoplasias in chronic ulcerative colitis Colorectal carcinoids of diameter < 20 mm (EMR, when diameter < 10 mm)	[5,10,11,16,64]

[1]Indications with risk of LNM < 1%; [2]Indications with risk of LNM or systemic M < 4%; [3]Increased risk for stricture formation, when ESD extends for ≥ 70% of circumference; [4]LST-granular type may also be resected in piecemeal fashion, the larger nodule resected first[10]; [5]SM1 invasion of ≤ 1000 μm. LNM: Lymph node metastasis; ESD: Endoscopic submucosal dissection.

Table 2 Criteria of curative endoscopic resection *en-bloc* in esophagus, stomach, and colorectum (modified from[17])

Stomach
Guideline criteria[1]
m-ca, diff. type, ly (-), v (-), and Ul (-) and ≤ 2 cm in size
Expanded criteria[2]
m-ca, diff. type, ly (-), v (-), Ul (-) and any size > 2 cm
m-ca, diff. type, ly (-), v (-), Ul (+) and ≤ 3 cm in size
sm 1-ca (invasion depth < 500 μm[3]), diff. type, ly (-), v (-)
m-ca, undifferentiated type (G3), ly (-), v (-), Ul (-) and size < 2 cm
Esophagus (squamous lesions only)
Guideline criteria[1]
pT1a-EP-ca/pT1a-LPM-ca
Expanded criteria[2]
pT1a-MM-ca, ly (-), v (-), diff. type, expansive growth, ly (-), v (-)
cT1b-sm-ca (invasion < 200 μm[3]), ly (-), v (-), infiltrative growth pattern, expansive, diff. type, ly (-), v (-)
Colorectum
Guideline criteria[1]
m-ca, diff. type, ly (-), v (-)
sm-ca (< 1000 μm[3]), diff. type, ly (-), v (-)

[1]Indications with risk of LNM < 1%; [2]Indications with risk of LNM or systemic M < 4%; [3]Measured as distance of maximum vertical invasion below MM. m: Mucosal; ca: Cancer; diff: Differentiated; ly: Lymphatic invasion; v: Vascular invasion; Ul: Ulceration; sm: Submucosal; EP: Epithelium; LPM: Lamina propria mucosae; MM: Muscularis mucosae.

for rectosigmoidal cancer (Table 3). During tumor staging with IEE, lateral extension of early Barrett´s adenocarcinoma and invasiveness of esophageal squamous cell cancer and rectosigmoidal cancer had obviously been underdiagnosed. Accordingly, the rate of surgical resection for noncurative ESD was too high, median 8% and up to 28%. In general, the learning curve for ESD still is flat even in the pioneering Western centers and performance not yet on the professional level of leading centers in Japan. The strategy must be to establish Western reference centers on comparable professional level as in Japan (Figure 1).

BACKGROUND AND PREPARATIONS FOR LEARNING ESD

Indications for ESD are pre/malignant superficial neoplasias without LNM. The risk of regional LNM rises from less than 5% to about 20% with increasing depth of vertical invasion into the submucosa (sm) layer, because lymphovascular supply increases deeper in the sm layer (sm2, sm3)[1,62,63]. Usual plane of dissection for ESD is the deeper third of the sm layer (sm3)[17]. Therefore, the probability of LNM has been determined in relation to the precise depth of sm invasion below the muscularis mucosae in very large series of differentiated early cancer (G1, G2) treated by surgical resection and lymphadenectomy. The maximum depth of superficial sm invasion consistent with minimum risk (< 4%) of lymph node metastasis is 500 μm in stomach, 1000 μm in colorectum, and 200 μm for esophageal squamous cell cancer with favourable prognostic indicators[1,8,13,64]. Evidence for massive sm invasion - deeper than this - is strict contraindication to endoscopic resection. Curative resection is reported when early cancer resected en-bloc reveals on serial sections differentiated carcinoma (G1/G2) without or with such extent of superficial sm

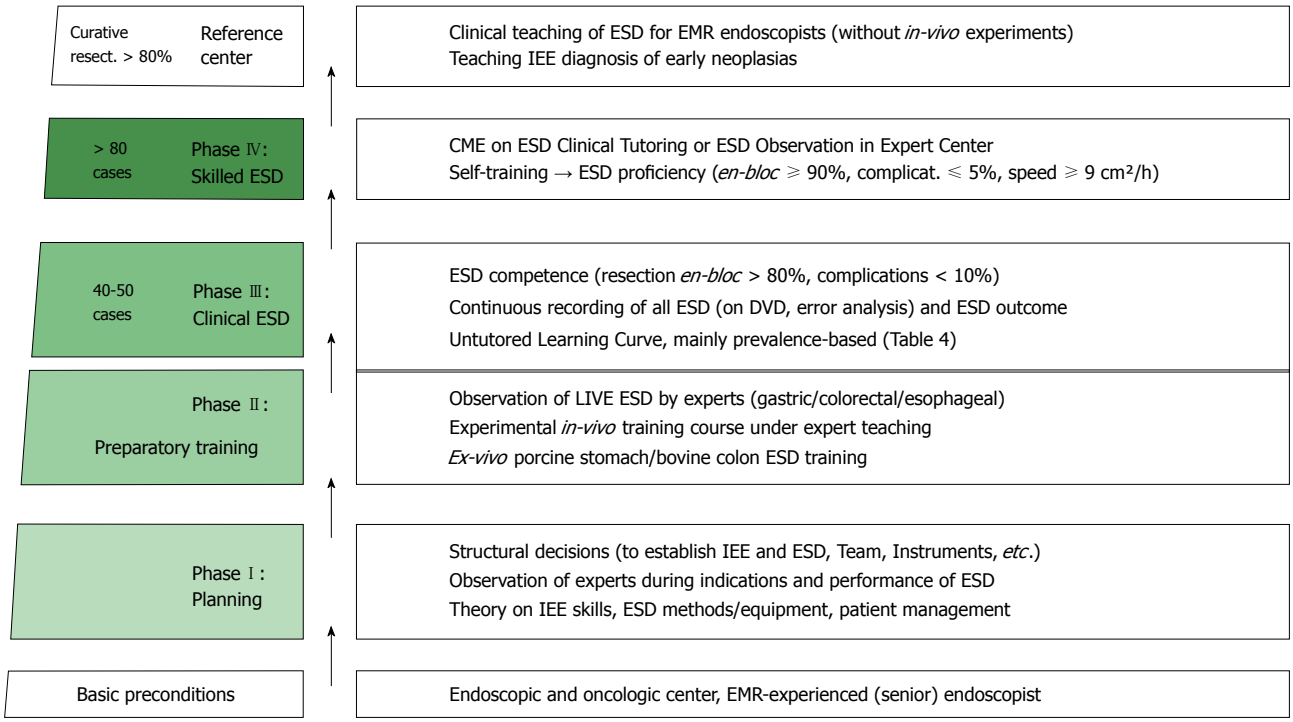

Figure 1 Strategy how to learn and establish endoscopic submucosal dissection in the west. ESD: Endoscopic submucosal dissection.

invasion without discontinuous cancer cell nests at the invasion front, without lymphovascular invasion (L0, V0) and resected R0 with tumor-free margins[65] (Table 2). Therefore, curative ESD depends on accurate endoscopic categorization of early neoplasias (*i.e.,* indication) as well as on dissection technique (*i.e.,* operation).

Endoscopic categorization of of early neoplasias with IEE

Analysis with IEE can estimate the tumor category of superficial neoplasias. Optimized conditions require adequate preparation of the patient including sedation, cleaning of the mucosa from adherent mucus, use of a 60- to 100-fold magnifying endoscope with distal attachment to keep optimum distance for focussing on the microsurface (depth of field 3 mm) and mode for virtual chromoendoscopy (NBI, FICE, i-scan) with optimized processor settings[7]. Endoscopic analysis of the microsurface and capillary pattern of mucosal neoplasias in the GI tract is complex and requires special knowledge recently collected in an endoscopy atlas[66]. The staging diagnosis is substantiated by the type of atypias of the surface pattern on magnifying white light chromoendoscopy, and the type of alterations of sub-/mucosal capillary pattern using spectral light of 415 and 540 nm wavelength (virtual chromoendoscopy)[7]. IEE competence is indispensible to accurately analyze early carcinomas for grading (differentiated *vs* undifferentiated), lateral extension (margins) and extent of invasion (mucosal or slightly *vs* massively sm-invasive). The details are beyond the scope of this minireview (compare[67-73]). Most of this knowledge has been contributed over the past 15 years by endoscopic researchers from East Asia, but not widely introcuced to Western countries because comparable routine scopes for magnifying IEE were not marketed in the West until 2013. However, diagnostic proficiency to categorize early GI neoplasias with IEE at > 90% accuracy is fundamental to make correct indications for ESD *vs* surgery and achieve curative ESD. This skill must be well trained during routine endoscopy and at national continued medical education (CME) programs in the West.

Knowledge on ESD performed by experts and structural decisions

ESD is the new discipline of *Endoluminal Surgery*. N. Yahagi called ESD a "low tech, but highly skilled procedure" for the following reasons: (1) single handed resection procedure by endoscope movement (lack of countertraction); (2) complex high-frequency electrosurgery; (3) tissue recognition and diagnosis in intramural layers; and (4) skilled team approach required (operator and assistant).

At this stage of Western experience, EMR-experienced senior endoscopists in endoscopic and oncologic centers should approach to establish ESD up to professional level. The procedure should be learned from Japanese experts. The first step on the individual electrosurgical learning curve is to acquire background knowledge on ESD procedures and carefully observe at least 15 procedures performed in different locations of the GI tract by professional experts in Japan (Figure 1). The following decisions must be based on this practical experience and theoretical background highlighted

Table 3 Organ-specific outcome of endoscopic submucosal dissection (curative intention) for Western prospective studies

Ref.	Malignant neoplasia type[6], *n*	ESD, *n*	Resection *en-bloc*, %	Resection curative[6], %	Complications, %	Surgery, %	Mortal., %	Recurrence, %	Follow-up (med.) yr	DFS, %/yr
Gastric ESD										
Cardoso *et al*[46], 2008	GC 15	15	80	74	20	8	0	8	1	91/1
Catalano *et al*[47], 2009	GC 12	12	92	92	16	8	0	8	2.5	92/2
Probst *et al*[49], 2010	GC 66	91	87	72	10.6	11	0	5.6	2.3	96.7/2
Schumacher *et al*[50], 2012	GC 21	28	90	64	20	7	3.4	11	2	100/2
Pimentel-Nunes *et al*[51], 2014	GC 128	136	94	82	13	7	0	7	3.2	100/3
median [range]			90 [80-94]	73 [64-92]	15 [11-20]	8 [7-11]	0 [3.4]	8 [5-11]	2.3 [1-3]	97 [91-100]/2
Esophageal ESD										
Repici *et al*[52], 2010	SCC 20	20	100	90	15	10	0	0	1.5	100/1.5
Neuhaus *et al*[53], 2012	AC 26	29	90	39	17	0	0	4	1.5	96/1.5
Arantes *et al*[54], 2012	AC 25	25	92	80	12	4	0	8	1.5	96/1.5
Höbel *et al*[56], 2014	AC 22	22	96	77	27	23	0	6	1.6	94/1.6
Chevaux *et al*[55], 2015	AC and HG 66	73	90	64	7 (+60[3])	10	0 (3[1])	(10[5])	1.8	92/2
Probst *et al*[57], 2015	AC 87	87	95	72 (84[4])	12.6	6	0 (2[1])	5	2.0	98/2
Probst *et al*[57], 2015	SCC 24	24	100	46 (72[4])	12.6	0	0 (4[1])	4	3.2	96/3
median [range]			95 [90-100]	72 [39-90]	16 [12-66]	6 [0-23]	0 [0-4]	4 [0-8]	1.6 [1.5-3.2]	96 [94-100]/2
Colorectal ESD										
Probst *et al*[59], 2012	Rectosigm. LST	76	82	-	9.2	15	0	n.g.	2.0	100/2
	14 CRC		86	7		(79[2])		0		
Iacopini *et al*[58], 2012	Colorectal LST	60	68	-	10	20	0	n.g.	n.g.	n.g.
	29 CRC			72	n.g.	(28[2])				
Repici *et al*[60], 2013	Rectal LST	40	90	-	7.5	5	0	2.5	0.5	100/0.5
	8 RC			75	n.g.	(25[2])				
Thorlacius *et al*[61], 2013	Colorectal LST	29	72	76	10	10	0	n.g.	< 0.5	n.g.
	10 HG and CRC			80		(20[2])				
Berr *et al*[38], 2014	Colorectal LST	39	76	-	17	3	0	LG 9	1.5	100/1.5
	12 HG		83	83		(0[2])		HG 0		100/1.5
median [range]			83 [72-90]	75 [7-83]	10 [7.5-17]	10 [3-20]	0	8 [2.5-9]	1.5 [0.5-2]	100/1.5

[1]Rate (%) due to cancer progression; [2]Surgery (%) for malignant lesion after ESD; [3]Plus stenoses (%); [4]Rate (%) of R0 resection; [5]Metachronous HGIN or cancer; [6]Curative resection does only apply for malignant neoplasias (cancer -/+ HGIN). AC: Adenocarcinoma; CRC: Colorectal carcinoma; GC: Gastric cancer; HG/LG: High/low-grade intraepithelial neoplasia; RC: Rectal carcinoma; SCC: Squamous cell cancer; DFS: Disease-free survival rate; n.g.: Not given; pmEMR: Piecemeal EMR.

in a recent textbook[74]: (a) The principal decision, whether to establish IEE and ESD competence in this hospital depending on existing experience with EMR and complication management, and on predicted case load (> 2 per month); (b) The subsequent decisions, how to assemble a team (operator, assistants, pathologist), select and provide best suitable equipment (special endoscopes with CO_2-insufflation and electrosurgical unit), instruments and devices, organize top maintenance of endoscopes, and finally which type(s) of electroknife to use for start-up[75]. Beginners most easily control tip knives with flexible shaft (dual knife, hook knife) that use for lifting of the submucosa (sm) layer separate sm-injection of suitable solutions with a 25 gauge needle[74]. In the beginning this is less challenging than knives with integrated injection system (flush knife or hybrid knife), for the simple reason to control only three pedals for water jet, cutting and coagulation modes, but not an additional pedal for sm-injection *via* knife. On the other hand, knives with integrated injection system allow to maintain a safer, permament submucosal liquid cushion by frequent reinjection. At this point, accurate staging of neoplasias with IEE and thorough theoretical knowledge of ESD technique, equipment, complications and their management must be attained.

WESTERN DEMAND FOR TRAINING IN ESD

Ex-vivo training systems should first be used to acquire team coordination (operator and assistant) and basic dexterity for proper positioning of the scope in relation to the lesion, correct maneuvres of sm-injection, marginal incisions, submucosal access with the transparent distal attachment of the scope for sm-dissection, and electrothermal knife techniques - adequate current modes ("cut, coagulation, blended"), impulse duration, voltage and Watt settings, correct short duration of application by pedal tap[76-79]. Approach to the lifted lesion is preferably in knife position tangential to the proper muscle layer. Avoid to cut in perpendicular position to proper muscle layer or haustral folds, in order to prevent inadvertent muscle layer perforation. Train how to use the effect of gravity to keep the vision field clear and facilitate access to the submucosal space for further dissection. Basic dissection strategies such as initial complete circumferential incision (icci), partial circumferential incision method (pci, to longer maintain sm-lifting)[6,74], and hybrid-ESD-snaring[80] should be practiced, as well as clip closure techniques of the resection bed for complication management[81]. Thorough preparation

Figure 2 Distribution of reference centers with interventional endoscopists participating in the seven experimental endoscopic submucosal dissection workshops [red flags, *n* = 110, (30 two times)] and in the Clinical endoscopic submucosal dissection-Tutorings (blue flags, *n* = 51, repeatedly). Additional participants in workshops and clinical tutoring from Jerusalem, Amman and Cairo, respectively. Created with www.google.com/maps.

of basic ESD techniques in *ex-vivo systems* - porcine stomach and esophagus and bovine colon[82-84] - contributes significantly to coordinated team work and training in individual ESD performance, and 20 to 30 procedures under preceptorship are sufficient to gain expertise[31,83,85,86].

Videotraining demonstrating typical high-risk maneuvers that have led to perforation or bleeding - compared to the correct strategy in that situation - could decrease complications during the early learning curve for untutored performance of clinical ESD, however is not yet available.

Experimental training in-vivo under expert supervision

In Western countries, training courses on *in-vivo* animal models generate additional progress after *ex-vivo* training[83,87-89]. Essential for optimal educational value is the expertise and teaching of the preceptors. For transition from *ex-vivo* training to clinical ESD, we recommend experimental training *in-vivo* - at least five gastric ESDs - under preceptorship of Japanese experts. This had been proposed in 2008 by T. OYAMA, because Japanese experts were not authorized to tutor trainees during ESD on patients in Western countries. After the first such expert training course in 2009, about two thirds of the participants increased their case load in gastric ESD (2.5-fold), colorectal ESD (3-fold), and esophageal ESD (8-fold) during the subsequent year at an acceptable rate of complications (9.7% perforations, 4.2% bleedings) without longterm morbidity[88]. Aims were propagation of technical skills, dissection maneuvers, specific electrocautery applications, strategy to keep a clear field of vision, and management of intentional complications (bleeding, perforation) in theory and practice. The experimental program and the personal tutoring by leading experts from Japan was ranked excellent by participating highly experienced endoscopists in their early learning curve, and therefore the course was repeated annually (Figure 2). Such a course is probably improving the outcome in the early untutored learning curve for ESD.

LEARNING CURVE IN ESD IN WESTERN COUNTRIES

Lesions suitable for initial learning of ESD must be strictly intramucosal, of moderate size (< 5 cm diameter) and in locations technically not very challenging[31,90]. Exclude any cancer with evidence for sm-invasive parts on IEE during the early learning curve, and avoid duodenal as well as very large (diameter > 6 cm) or very fibrotic lesions (*e.g.*, recurrent neoplasias). However, the risk of inadequate oncological treatment due to an artefactual incomplete

Table 4 Principles for establishing endoscopic submucosal dissection by an untutored learning curve (modified from[38])

Evaluate the lesion during prior endoscopy for ESD indication and resection strategy
Avoid risk of any R2 resection of cancer (no signs for deep submucosal invasion!)
Avoid high risk lesions (> 5 cm diameter, or in fornix and cardia, duodenum, colonic flexures)
Safety comes first, procedure time of ESD is of minor importance in the beginning
Only cut tissue or fibers in submucosa that you clearly see and have identified
Keep the vision field clear, prevent and immediately stop bleeding
Close any perforation immediately by endoscopic clipping on expert level
Complete any started ESD procedure with intention for safe, curative resection
Guide personally the patient pre-ESD (informed consent) and post-ESD (for any complication)
Only a single endoscopist per unit should do untutored ESD until he is on competence level[1]
Document all entire ESD procedures on DVD recordings (for evidence and error analysis)
Follow-up short-term and long-term (center Registry), trend in dozens

[1]Performance of 20 consecutive ESD procedures with < 10% complications. ESD: Endoscopic submucosal dissection.

resection R1 is high during the initial learning curve[31], and may be more dangerous than minor perforation managed well by proficient clipping. Undertreatment (R1 resection) results in major resective surgery or high risk of recurrence and incurable disease[31,90]. Therefore, early cancer lesions in esophagus and upper half of stomach should be reserved for proficient endosurgeons, and not treated before the competence level for that organ has been accomplished[31,39]. This suggests that large part of untutored initial learning for ESD may better be passed on rather challenging adenomatous/dysplastic LST´s in the colorectum than on early cancer in stomach or esophagus[38].

Strategy for untutored learning of ESD

In 2008, a panel of experts had recommended a "step-up approach" in technical challenge for untutored learning of clinical ESD[90]. The first 20 ESD should be performed on neoplasias in the antrum and distal corpus of stomach, and in rectum, before more challenging locations are approached. This strategy has been very successful for tutored training in ESD in Japan, where however gastric cancer has 10 fold higher incidence and is more often detected as early cancer (in 70% *vs* 20%-30%) than in most Western countries[31,39,91]. Therefore, early gastric cancer is too rare in the West to achieve a useful case load of at least two ESD procedures per month. This recommendation would impede to establish ESD for decades - to the disadvantage of GI cancer patients. Alternatively, a "*prevalence-based approach*" allows for a reasonable case load, but requires learning ESD mainly in the colorectum and early on in difficult locations[38,91]. Such an approach has successfully been taken after the described basic preparations and experimental *in-vivo* training in gastric ESD supervised by Japanese experts[38,88].

Untutored learning of ESD

The risk of complications is highest during the early untutored learning curve[44]. We recommend that only the most skilled and EMR experienced endoscopist of the unit undertakes to establish ESD in the early untutored learning curve[31,38,39]. After rigorous theoretical and experimental preparation, a skilled interventional endoscopist can achieve competence level after 20 to 30 untutored ESD procedures, and needs twice that case load (*e.g.*, 2 x 25 ESD) to prove outcome for competence level[38,59]. The outcome for untutored colorectal ESD without significant experience in gastric ESD[38] was quite similar as reported with the step-up approach by others[35,59,92]. However, this should not be endeavoured with little interventional expertise and low theoretical background. Meticulous preparation is important for any of those untutored ESD procedures (Table 4). In addition, close personal contact with the patient is essential before ESD for fully informed consent and after ESD to monitor/treat complications and in the long-term to detect and handle any local recurrence or delayed complication such as stenosis. The coo-perating pathologist must receive IEE information about suspicious areas in the oriented specimens, and provide precise histologic work-up for tumor grading, sm invasiveness, lymphovascular infiltration and resection status - curative resection is critical for the patient[65]. Continuously register outcome quality to evaluate the level of performance and spur to improve ESD technique[38]. ESD on beginning proficiency level (*en-bloc* 90%, complications < 5%, curative resection > 80%, speed about 9 cm^2/h) requires more than 100 self-completed procedures[31,35,85,93] and continued education by and feed-back with top experts.

CONTINUED MEDICAL EDUCATION IN IEE AND ESD

Continued expert instruction for ESD

Nevertheless, immediate preceptorship of trainees by proficient ESD experts best guides through the early learning curve for ESD. This was the key for enormously rapid spreading of ESD throughout Japan. Intense observation of experts performing ESD (about 40 cases) can enhance performance of ESD during the clinical learning curve. At present this requires a sabbatical of four weeks in Japan. When combined with some *ex-vivo* technical training, performance and skills markedly increase during subsequent untutored ESD, as shown by doubling of dissection speed[39]. ESD clincal tutoring program was introduced by four top experts from Japan together with eight endoscopists

in the ESD learning curve, former participants of the Experimental ESD Training Workshop[88], in order to enhance progression to professional level both in IEE categorization of mucosal neoplasias and ESD performance (Figure 2). The educational benefit is immediate preceptorship during the diagnostic and endosurgical procedures, assessment of differential indication and risk for ESD, tipps and tricks for strategy and technique of dissection for different lesions, preventive hemostasis and/or clipping of the resection bed, and documentation of the specimen for histopathology. The enrolled patients received excellent treatment, as shown by the outcome of a series of 116 ESD performed intention-to-treat in cooperation with expert tutors from Japan. The curative resection rate of all 49 malignant neoplasias and five symptomatic semimalignant submucosal tumors was an astonishing 100% at an acceptable rate of complications (14%) managed without surgery or long-term morbidity in these elderly, often comorbid patients[94]. We highly recommend such CME with Japanese experts for establishing ESD in Western countries.

ESD reference centers where ESD is performed on a professional level as high as in Japan must arise in Western countries to spread ESD to all referral centers (Figure 1). Expert instructors in ESD reference centers can better guide advanced trainees for ESD how to increase safety, dexterity (*en-bloc* resection, speed), specimen quality, and outcome (curative resection, low complication rate). These centers must take a leading role in national CME programs for IEE and ESD of early neoplasias.

Medical progress and patients rights

Diagnosis with IEE and performance of EMR/ESD/surgical resection with lymphadenectomy according to the criteria established in Japan is state-of-the-art for early neoplasias in the gastrointestinal tract (Table 1)[4,5,18], but requires proficient performance of diagnostic IEE and electrosurgical ESD with curative outcome. The benefit for patients with such early neoplasias is so enormous that major endoscopic referral hospitals have the duty to establish ESD. Typically, this should be a third level endoscopic referral center that manages a high volume of neoplastic lesions allowing a suitable case load of more than two ESD indications per month. Establishing ESD in such centers should be restricted to the endoscopist most experienced with EMR, emergency endoscopy and IEE staging of early neoplasias[39]. In many Western countries, performance of ESD by such a leading endoscopist under tutoring and, if necessary or better for the patient, with direct help by the Japanese expert, is permissible and compatible with legal and insurance rules. For untutored ESD in the learning phase, the indication must conform to criteria established in Japan and national guidelines, and in any doubt expert advice should be obtained *via* web-based image and video analysis. All lesions comprising early cancer must be presented to an interdisciplinary cancer board prior to ESD and again with the histopathological results. For advanced adenoma (*e.g.,* in colorectum) the decision can be made by patient and treating physician after informed consent on ESD and alternative resection techniques, including the lower level of technical performance of ESD as compared with data from Japan. Since individual learning curves are involved, we recommend detailed documentation of all entire procedures with DVD recordings (for evidence and error analysis) and a continuously updated registry of the outcome data (procedure, complications, histopathology, follow-up) that also enhances the learning process.

CONCLUSION

To establish accurate endoscopic diagnosis and endosurgical treatment of early cancer in Western countries lasts longer than anticipated five years ago, but the strategy to achieve it is quite clear (Figure 1). IEE of early GI neoplasias must become daily practice and part of national CME programs. EMR-experienced senior endoscopists from major endoscopic referral centers ought to establish ESD on competence and subsequently proficiency level. They need to understand the present lack of competence and the long-lasting learning curve. When knowledgeable about IEE and ESD, they have to negotiate with the hospital for sustained funding of team and optimal equipment for IEE and ESD. Dedicated *ex-vivo* training must be combined with experimental ESD training *in vivo* supervised by experts from Japan before untutored performance of first clinical ESDs. Continued instruction by experts from Japan is highly recommended until proficiency in ESD of early GI cancer is confirmed by outcome data. Western experts in new reference centers must commit themselves to teach EMR-experienced senior endoscopists from other referral centers in order to spread ESD. ESD is high-end endoscopic patient care that will be largely confined to referral centers.

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TOPIC HIGHLIGHT

2015 Advances in *Helicobacter pylori*

Diagnosis of *Helicobacter pylori* infection: Current options and developments

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Abstract

Accurate diagnosis of *Helicobacter pylori* (*H. pylori*) infection is a crucial part in the effective management of many gastroduodenal diseases. Several invasive and non-invasive diagnostic tests are available for the detection of *H. pylori* and each test has its usefulness and limitations in different clinical situations. Although none can be considered as a single gold standard in clinical practice, several techniques have been developed to give the more reliable results. Invasive tests are performed *via* endoscopic biopsy specimens and these tests include histology, culture, rapid urease test as well as molecular methods. Developments of endoscopic equipment also contribute to the real-time diagnosis of *H. pylori* during endoscopy. Urea breathing

test and stool antigen test are most widely used non-invasive tests, whereas serology is useful in screening and epidemiological studies. Molecular methods have been used in variable specimens other than gastric mucosa. More than detection of *H. pylori* infection, several tests are introduced into the evaluation of virulence factors and antibiotic sensitivity of *H. pylori*, as well as screening precancerous lesions and gastric cancer. The aim of this article is to review the current options and novel developments of diagnostic tests and their applications in different clinical conditions or for specific purposes.

Key words: *Helicobacter pylori*; Diagnosis; Invasive; Noninvasive; Oral specimen; Bleeding; Gastrectomy; Eradication

Core tip: Nowadays, several tests are available for the diagnosis of *Helicobacter pylori* (*H. pylori*) infection. In this review, we focus on the usefulness and limitations of current diagnostic methods as well as the recent developments of these tests that contribute to improve the diagnostic accuracy. Furthermore, we also emphasize the detection of *H. pylori* in oral specimens and in patients with different clinical circumstances, including bleeding, post-gastrectomy and post-eradication therapy.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a Gram-negative, microaerobic human pathogen and *H. pylori* infection is strongly related with many gastroduodenal diseases including chronic active gastritis, peptic ulcer diseases, atrophic gastritis, mucosa associated lymphoid tissue (MALT) lymphoma and noncardia gastric cancer. *H. pylori* infection affects more than half of the adult population worldwide, but the prevalence of *H pylori* infection varies widely by geographic area, age, race, and socioeconomic status. Usually, the prevalence of *H. pylori* increases with age in most countries, however a decline in prevalence of *H. pylori* infection has been observed in recent decades in time trend analysis of several large populations[1]. More than 80% of peptic ulcer diseases are caused by *H. pylori* infection and the estimated lifetime risk for peptic ulcer disease in *H. pylori*-infected patients is approximately 15%[2]. Gastric cancer is the third leading cause of cancer-related death worldwide and *H. pylori* infection is responsible for 74.7% of all noncardia gastric cancer cases[3,4]. Gastric cancer and peptic ulcer together cause more than a million deaths per year in the world and *H. pylori* infection always is an important health issue[5]. Various diagnostic methods are developed to detect *H. pylori* infection and diagnostic tests with both high sensitivity and specificity, exceeding 90%, are necessary for accurate diagnosis of *H. pylori* infection in clinical practice. Although many diagnostic tests are available now, each method has its own advantages, disadvantages, and limitations. The choice of one method or another could be depended on availability and accessibility of diagnostic tests, level of laboratories, clinical conditions of patients, and likelihood ratio of positive and negative tests on different clinical circumstances. Diagnostic tests are usually divided into invasive (endoscopic-based) and noninvasive methods. Invasive diagnostic tests include endoscopic image, histology, rapid urease test, culture, and molecular methods. Non-invasive diagnostic tests included urea breath test, stool antigen test, serological, and molecular examinations. In the present article, we briefly review the current options and developments of diagnosis tests and associated applications in clinical practices, as well as choice of diagnostic tests on different clinical conditions (Table 1).

INVASIVE TESTS

Endoscopy

Conventional endoscopic exam is usually performed to diagnose *H. pylori*-associated diseases, such as peptic ulcer diseases, atrophic gastritis, MALT lymphoma and gastric cancer. Endoscopy is also an instrument routinely used to obtain specimens, usually gastric mucosa from biopsy, for further studies on other invasive tests, including rapid urease test, histology, culture, and molecular methods. Antrum is a preferential biopsy site for detecting *H. pylori* infection in most circumstances, but corpus biopsy from greater curve is suggested for patients with antral atrophy or intestinal metaplasia to avoid false negative results[6,7]. The uneven distribution of *H. pylori* in the stomach in different clinical setting inevitably leads to sampling errors in biopsy-based examinations and several attempts have been made for real-time diagnosis of *H. pylori* infection during endoscopic examination.

Most gastric mucosal features, such as redness, mucosal swelling or nodular change, from conventional endoscopy are not specific enough for diagnosis of *H. pylori* infection and provide limited value in the accurate diagnosis[8]. Although careful close-up observation of the gastric mucosa pattern with standard endoscopy may increase the diagnostic accuracy, but it may be time-consuming and not provide better results than other invasive tests[9]. In additional to conventional endoscopy, chromoendoscopy with phenol red has also been evaluated for diagnosis of *H. pylori*

Table 1 Diagnostic options of *Helicobacter pylori* infection in different clinical circumstances and special applications of diagnostic tests

	Gastroduodenal bleeding	Post gastrectomy	Post eradication therapy	Special applications
Rapid urease test		√		
Histology		√		
Culture				√Antibiotic sensitivity
Polymerase chain reaction	√		√	√Antibiotic sensitivity √Virulence factors √Environmental/oral sample
Urea breath test	√		√	
Stool antigen test			√	
Serology[1]	√	√	√	√Virulence factors

[1]Although serology is not affected by local change in stomach, result of serology should be interpreted with caution before further management.

infection under the basis of specific urease activity of *H. pylori*. However, this method is not a reliable test because of its low sensitivity (73%-81%) and low specificity (76%-81%)[10,11]. Magnifying endoscopy provides direct observation of surface microstructure in the gastric mucosa and high resolution endoscopic patterns of gastric mucosa is highly correlated with histopathological changes, including *H. pylori* infection. The sensitivity and specificity for predicting *H. pylori*-positive corporal gastritis by using magnifying endoscopy with indigo carmine staining were 97.6% and 100% respectively. However the sensitivity and specificity decreased to 88.4% and 75.0% respectively in *H. pylori*-positive antral gastritis[12]. Confocal laser endomicroscopy (CLE) is the other magnifying endoscopic technique which provide subsurface analysis and *in vivo* histology examination of gastric mucosa during endoscopy. Three features including white spots, neutrophils and microabscesses, based on CLE findings, were used for *H. pylori* diagnosis and the accuracy, sensitivity and specificity were 92.8%, 89.2% and 95.7% respectively[13]. Magnifying narrow band imaging and I-scan were also used to detect *H. pylori* infection, but variable results were presented[14-16]. Different classifications of image features from magnifying endoscopy provide different diagnostic accuracy and the accuracy of endoscopic test is also operator dependent, which means its use require training process from experienced supervisor and availability of equipment from local endoscopy unit[17-20]. Moreover, careful examination by using magnifying with or without image-enhanced technique is also time-consuming and may make more discomfort to patient than other biopsy-based tests. Those factors usually limit the clinical use of magnifying endoscopy to detect *H. pylori* infection in routine practice.

Histology

Histology is usually considered to be the gold standard in the direct detection of *H. pylori* infection and is also the first method used for the detection of *H. pylori*. However, several factors influence the diagnostic accuracy of histology, such as site, size and number of biopsies, staining methods, proton pump inhibitor (PPI), antibiotics and experience of the examining pathologist. PPI use may lead to controversial results of histological exam and stopping PPI 2 wk before performing histological test is recommended[21]. More biopsy samples collected from appropriate site for analysis can decrease sampling error and false negative results in histological test as well as other biopsy-based tests. Biopsies from both antrum and corpus are usually recommended in clinical practice and the acquisition of at least two biopsy specimens from antrum and corpus is a most sensible strategy that guarantees the maximum diagnostic yield[22,23]. As mentioned above, corpus biopsy is important for the diagnosis of *H. pylori.* in a background of atrophic gastritis[7].

Staining is the critical part of histological exam and several stains like routine HE staining, Giemsa, Warthine-Starry, Hp silver stain, toluidine blue, acridine orange, McMullen, Genta, Dieterle, and immunohistochemical stain have been used to detect *H. pylori*. Although immunohistochemical stain is the most sensitive and specific stain, HE stain is usually sufficient for diagnosis of *H. pylori* infection in routine clinical practice. Ancillary stain is usually recommended for biopsy specimens which revealed moderate or severe chronic gastritis, but no *H. pylori* identified in HE staining. Furthermore, immunohistochemical stain should be the first choice if ancillary stain is decided to use for detecting *H. pylori*[24,25]. If immunohistochemical stains are not available, Giemsa stain is the preferred method in clinical practice because it is simple, highly sensitive and less expensive[26].

Peptide nucleic acid fluorescent *in situ* hybridization (PNA-FISH), which can be used on histological preparations, is a highly sensitive (97% sensitivity) and specific (100% specificity) technique for the diagnosis of *H. pylori* infection. PNA-FISH can identify coccoid form of *H. pylori* which is usually undetectable by routine histological exam because this method could avoid individual biasness from morphological identification. Moreover, PNA-FISH is a rapid, accurate and cost-effective method for detection of *H. pylori* clarithromycin resistance in gastric biopsy specimens[27-29]. FISH also has the potential role in the

detection of *H. pylori* in environmental samples and further studies on the transmission and environmental reservoirs of *H. pylori* could be conducted by using FISH[30,31]. Despite the advantages of detection of *H. pylori* and clarithromycin resistance at the same time, the disadvantages of PNA-FISH, such as laborious prepare, requiring fluorescent microscope and particular expertise to read the slides, may limit the broadly use of this method.

RAPID UREASE TESTS

For routine clinical practice, rapid urease test (RUT) is the most useful invasive test for the diagnosis of *H. pylori* infection because it is inexpensive, rapid, easy to perform, highly specific and widely available. Based on the activity of the *H. pylori* urease enzyme, the presence of *H. pylori* in biopsy specimen convert the urea test reagent to ammonia, leading to an increase in the pH and a color change on the pH monitor. Several commercial urease tests including gel-based tests (CLOtest, HpFast), paper-based tests (PyloriTek, ProntoDry) and liquid-based tests (UFT300, EndoscHp) are available now, and different commercial RUTs have different reaction time to provide results. CLOtest usually takes 24 h to obtain accurate result, whereas PyloriTek takes 1 h and UFT 300 takes 5 min to provide more rapid results. Reading the urease tests earlier than recommended time may lead to false negative results[32]. In addition to the designs of commercial kits, the density of bacteria present in the biopsy specimen also affects the reaction time and diagnostic accuracy of RUT, while the minimum of 10000 organisms are usually required for a positive RUT result. Other factors influencing the diagnostic accuracy of the urease tests include H_2-receptor antagonists, PPI, bismuth compounds, antibiotics, achlorhydria and presence of blood, all of which increase the possibility of false negative results. Furthermore, formalin contamination of biopsy specimens also decrease the sensitivity of RUTs[21,33-35].

In general, the commercial rapid urease tests have specificity above 95%-100% and sensitivity above 85%-95%. Increasing the number of gastric antral biopsies could increase the sensitivity of RUTs and dual biopsy specimens from gastric corpus and antrum are preferred than only antrum biopsy specimens as additional corpus biopsy increase the diagnostic accuracy and avoid sampling bias due to uneven distribution of *H. pylori* in stomach. Moreover, combining antrum and corpus specimens prior to RUT, rather than separate specimens, also increased the sensitivity of RUT and accelerate the reaction time[32,36-39]. Avoid medications that affect the urease activity and the density of bacteria is recommended before RUT to decreased false negative results, such as 2 wk for PPI and 4 wk for antibiotics. Bleeding significantly decreases the sensitivity and specificity of RUTs and make RUT become a more unreliable test than other tests in this clinical condition[40]. In a study evaluated the influence of different biopsy number and site on results of RUT in patients with peptic ulcer bleeding demonstrated that four biopsies from antrum or one biopsy from body increased the sensitivity of RUT as compared with only one biopsy from antrum. In this study, sensitivity of one biopsy from antrum was 64%, whereas sensitivity of four biopsies from antrum and one biopsy from body were 74% and 73% respectively[41]. If RUT is still chosen for patient with gastrointestinal bleeding, biopsies from both antrum and corpus were suggested to increase the diagnostic accuracy.

Culture

Culturing of *H. pylori* from gastric biopsy specimen is a highly specific but less sensitive method. In general, culturing has almost 100% specificity, but the sensitivity of culture shows significant variation, between 85%-95%. Because of the delicate and fastidious nature of *H. pylori*, the cultivation in vitro requires particular transport medium, growth medium and incubation environment. Biopsy specimens can be kept in a transport medium, like Portagerm pylori or Stuart's transport medium, for up to 24 h at 4 ℃. Several types of agar can be used for culture as *H. pylori* are isolated. The commonly used media include Pylori agar, Skirrow agar, Columbia blood agar, Brucella agar, Brain heart infusion or Trypticase soy agar, supplemented with sheep or horse blood. The agar plates are usually incubated in a microaerobic environment (80%-90% N_2, 5%-10% CO_2, 5%-10% O_2) at 35 to 37 ℃ for at least 5-7 d because *H. pylori* has been considered a microaerophile. However, a recent study showed growth of *H. pylori* is promoted by atmospheric oxygen levels with the presence of 10% CO_2, bringing a novel concept that *H. pylori* may be a capnohilic aerobe[42]. Diagnosis of *H. pylori* from culture medium is based on morphological characteristics as well as positive urease, catalase, and oxidase reactions, which mean the microbiological laboratories should be equipped and trained to isolate this bacterium.

Conditions such as poor quality of specimens, delayed transport, exposure to aerobic environment or inexperienced microbiologist have adverse influence on the performance of culture and reduce the diagnostic accuracy[43]. A recent study conducted in 26 hospitals to analyze the influence of transport time as well as temperature on culture rate showed positive culture rate decreased to 26.3% in 48 h transport group as compared to 32.8% in 24 h transport group ($P < 0.001$). This study also found the average temperature increased from 4.7 ℃ to 29.1 ℃ during transportation and this caused positive culture rate declined from 36.7% to 24.1%[44]. The recent development of transport medium is a new transport medium, GESA transport medium. GESA transport medium is a semi-solid medium which can store gastric biopsy specimens

at 4 ℃ for up to 10 d and provide a quantifiable recovery rate of *H. pylori* (90.7%)[45]. A new biphasic test which combined the selective enrichment broth and biochemical test using urea agar in a single vessel was also developed for culturing *H. pylori* in gastric biopsies. In this small study, biphasic test was conducted in 55 biopsy specimens and showed 100% positive predictive valve after 48 h incubation. Moreover, this method had lower false positive rate and required lower bacterial load, approximately 10^5 cfu/mL, as compared with CLOtest. At the same time, this test could be used under an aerobic condition and allowed culturing as well as antibiotic susceptibility testing[46].

Host factors like high activity of gastritis, low bacterial load, bleeding, alcohol drinking, and use of H_2- receptor antagonists, PPI, antibiotics have adverse effect on culture positive rate. These medications, except for antibiotics which should be avoided at least 4 wk, were also suggested to be avoided 2 wk before culture. To avoid sampling bias from the patchy distribution of *H. pylori* in stomach, at least 2 biopsy specimens from the antrum and 2 biopsy specimens from corpus were also recommended[47,48].

Although culture is a time-consuming, expensive and laborious test for *H. pylori* diagnosis, the antibiotic sensitivity test of *H. pylori* provided by culture is a particular advantage in clinical practice. As recommends from Maastricht IV Consensus Report, *H. pylori* culture and antibiotic susceptibility testing should be performed if primary resistance to clarithromycin is higher than 20% in a given geographical area or after failure of second-line treatment[21]. Furthermore, culture also allows isolation of *H. pylori* for further analysis of phenotypic and genotypic characterization to have better understanding of the pathogens and, consequently, offer therapy evaluation. With the increasing prevalence of antibiotic resistance, culturing is still a reliabe method for managing *H. pylori* treatment failure as well as surveying antibiotic resistance in population-based studies before other molecular tests are more widely available.

Polymerase chain reaction

Since the application of polymerase chain reaction (PCR) to detect *H. pylori* infection, PCR has been used extensively for the diagnosis of *H. pylori* from gastric biopsy specimens, saliva, stool, gastric juice and variable specimens. PCR provides excellent sensitivity and specificity, greater than 95%, as compared with other conventional tests and has more accurate results of detecting *H. pylori* in patients with bleeding. Several target genes including *UreA*, *glmM*, *UreC*, *16S rRNA*, *23S rRNA*, *HSP60*, and *VacA* genes, had been used for detection of *H. pylori* and using two different conserved target genes can increase the specificity, which in turn avoids false positive result, especially for samples other than gastric biopsy specimens. The other advantages of PCR, including fewer bacteria required in sample, faster results, and no need for special processing supplies or transportation, enable clinicians to make quicker and more accurate decision on patient's treatment. Furthermore, PCR also allows concurrent detection of specific mutations leading to antibiotic resistance, such as macrolide- and fluoroquinolone-resistance, and virulence factors, such as CagA and VacA[49-51].

As compared with agar dilution method (Etest) which is usually regarded as gold standard of antibiotic susceptibility test, real-time PCR (RT-PCR) had several advantages. First, using formaldehyde-fixed paraffin-embedded gastric tissue in PCR test is more convenient, rapid and sensitive than using fresh biopsy specimen in Etest, moreover, in this setting, RT-PCR also showed not inferior results of antibiotic susceptibility testing than Etest. In addition, PCR is more reliable to detect heteroresistant status which often cause false negative result in Etest, consequently, PCR can provide more accurate information for clinicians before starting antibiotic treatment[52]. A recent study that used RT-PCR in formalin-fixed paraffin-embedded samples to detect *H. pylori* infection and associated clarithromycine-resistance status investigated the efficacy of genotypic resistance-guided quadruple therapy as the first-line treatment for 385 patients with functional dyspepsia. In this study, 136 patients (35.3%) were diagnosed with *H. pylori* infection and the sensitivities of RT-PCR and histological examinations were 95.6% and 69.9% respectively. Quadruple therapy with bismuth potassium citrate, rabeprazole, amoxicillin, and clarithromycin was used for genotypically sensitive patients, in contrast, genotypically resistant patients were treated with bismuth potassium citrate, rabeprazole, amoxicillin, and furazolidone. Authors found the eradication rates were 100% for patients with clarithromycin-susceptible *H. pylori* and 94% for patients with clarithromycin-resistant *H. pylori* respectively for per-protocol analysis[53]. Second, RT-PCR is also a convenient method for epidemiological study on regional antibiotic resistance rate as a guidance for first-line empirical treatment. Furthermore, RT-PCR can detect the point mutations that cause antibiotic resistance as well as find the change of point mutation or occurrence of new mutation, which provide additional information for epidemiological studies and molecular research on genotype-phenotype relationships. Due to the possible change of mutations that cause antibiotic resistance with time, defining more than 5 point mutations when using PCR-based methods is important to achieve good accuracy in detecting antibiotic resistance[54-56].

The genetic mutations causing resistance to clarithromycin (23S rRNA), quinolones (*gyrA* gene), tetracycline (16S rRNA), rifabutin (*rpoB* gene) and amoxicillin (*pbp-1a* gene) have been described in previous studies and several commercial kits such as MutaREAL *H. pylori* kit, ClariRes real-time PCR assay and Seeplex ClaR-*H. pylori* ACE

detection system are available for the detection of clarithromycin resistance[57]. However, the precise mechanism of metronidazole resistance is less clear and the susceptibility genes such as rdxA and frxA have been implicated in previous studies with debated results. A recent study using Illumina next-generation sequencing to search candidate mutations for metronidazole resistance. This study confirmed mutations in rdxA gene had the major role in metronidazole resistance of *H. pylori* and mutations in frxA gene could enhance the metronidazole resistance only in the presence of rdxA mutations. Additionally, a new discovery of mutations in rpsU gene may have a role in metronidazole resistance to explain the metronidazole-resistant strains without the mutations in *rdxA* and *frxA* genes[58]. GenoType HelicoDR assay is a molecular test that combine PCR and hybridization, allowing the molecular defecation of *H. pylori* as well as clarithromycin and fluoroquinolones resistance within 6 h. In previous studies, the GenoType HelicoDR assay using bacterial strains or gastric biopsy specimens is highly accurate for clarithromycin resistance with 94%-100% sensitivity and 86%-99% specificity respectively; the GenoType HelicoDR assay is also accurate for fluoroquinolone resistance with 83%-87% sensitivity and 95%-98.5% specificity respectively as compared to the culture-based method[59,60]. However, a recent study evaluated the clinical usefulness of GenoType HelicoDR in Korea showed the sensitivity and specificity for clarithromycin resistance were only 55.0% and 80.0% respectively. The GenoType HelicoDR was also not accurate for fluoroquinolone resistance, showing the sensitivity and specificity were 74.4% and 70.0% respectively. The clinical applicability of GenoType HelicoDR in determination of antibiotic resistance may have some limitations which need further evaluations[61]. RT-PCR is conventionally used to quantify the *H. pylori* DNA in biopsy specimens, but performing RT-PCR can be a problem for clinical laboratories because of expensive thermocyclers. A dual-priming oligonucleotide (DPO)-based multiplex PCR was developed to detect both *H. pylori* infection and clarithromycin resistance and this test can be performed in any conventional thermocycler that costs less than RT-PCR. With a particular DPO primer design to amplify the *H. pylori* 23S rDNA and to detect the most common mutations, A2142G and A2143G, conferring clarithromycin resistance, DPO-PCR was proved to be rapid and accurate for *H. pylori* diagnosis and determination of clarithromycin susceptibility by using gastric biopsy specimens[62,63]. Furthermore, a recent study using tissue samples that had been processed by RUT to evaluate the diagnostic accuracy of DPO-PCR showed DPO-PCR had higher sensitivity than RUT and histology, and DPO-PCR could detect *H. pylori* infection in RUT-negative samples, meaning that this test can decreased the false negative result and reduce the need for re-endoscopic examination. The concordance rate of DPO-PCR between gastric biopsy samples and samples proceeded by RUT was 94.4%[64].

Detection of virulence factors by PCR helps to evaluate the genetic variation within virulence factors of *H. pylori* and gives more information to understand the clinical discrepancies between patients infected with different strains of *H. pylori*. Several studies showed presence of virulence factors, such as CagA and *VacA* gene, are associated with more severe gastric inflammation and higher prevalence of peptic ulcer disease and gastric cancer[65-67]. Duodenal ulcer promoter gene A (DupA) was also proposed to be associated with *H. pylori* induced ulcer formation, but inconsistent results which were suspected to be caused by primer mismatches were reported by previous studies. A newly designed RT-PCR with a specific primer designed based on an alignment of all 221 DupA gene sequences was introduced recently to improve the detection rate of the DupA gene. This method increased the detection rate to 64.2%, whether the commonly used PCRs had detection rate between 29.9% to 37.8%. The authors pointed out that PCR design had great influence on the detection of virulence factor and the detection of specific DupA allele was not the same as detection of actual DupA gene[68].

PCR is also helpful to detect *H. pylori* in environmental samples for epidemiological studies. A high prevalence of *H. pylori* detected in drinking water samples by PCR provided more information of *H. pylori* transmission through drinking water[69]. Higher detection rate of *H. pylori* contamination in un-washed vegetable suggested accurate washing of vegetables decreased *H. pylori* contamination[70]. PCR had also been used to detect genotyping of *H. pylori* in vegetable and high similarity in the genotyping pattern of *H. pylori* among vegetable samples and human specimens suggested that vegetable may be the sources of the bacteria[71].

Except for more rapid and highly accurate results from PCR to detect *H. pylori* infection and antibiotic-resistance strains, concerns about cost, local available equipment and expertise in molecular techniques inevitably influence the feasibility of PCR in local laboratories.

NONINVASIVE TESTS

Several attempts have been made to avoid endoscopic diagnostic methods for several reasons. First and foremost, endoscopy is an invasive procedure which is discomfort and not suitable for patients with severe comorbidities or contraindications. Besides, cost of endoscopy and additional cost adding on endoscopy, such as disposable forceps and anesthesia, may be high. Last but not least, sampling bias is almost inevitably encountered in biopsy-based methods due to uneven distribution of *H. pylori* in stomach.

UREA BREATH TEST

Urea breath test (UBT) has been used for almost 30 years and is still the most popular and accurate noninvasive test for diagnosis of *H. pylori* infection. By the urease activity of *H. pylori*, the ^{13}C- or ^{14}C-labeled urea ingested by the patient is hydrolyzed to labeled CO_2 in stomach, then labeled CO_2 is absorbed in the blood and exhaled by breathing in which labeled CO_2 can be measured. Although several factors including patient, bacteria and the test itself influence the results of UBT, the UBT is a highly accurate and reproducible test with near 95% sensitivity and specificity under standardized procedures. A recent publish meta-analysis to evaluate the diagnostic accuracy of UBT in adult patients with dyspeptic symptoms showed the pooled sensitivity was 96% (95%CI: 0.95-0.97) and pooled specificity was 93% (95%CI: 0.91-0.94)[72]. UBT is also useful for epidemiological studies and for assessing the efficacy of eradication therapy[21,73]. Patient should stop taking PPI 2 wk and antibiotic 4 wk before exam to avoid false negative results[74]. Bleeding also influences the diagnostic accuracy of UBT and delayed UBT after recovery from bleeding is mandatory to decrease false negative result[75]. Sometimes, although rare, the presence of other urease producing pathogens in stomach also causes the false positive results.

UBT is a suitable method with many advantages, such as simple, noninvasive and safe, to detect *H. pylori* infection in pediatric patients, although the accuracy of UBT in pediatric patients is not as good as it used in adult patients, especially for children younger than 6 years old, having 75% to 100% sensitivity and specificity[76].

^{13}C-UBT is preferable to the ^{14}C-UBT to avoid exposure to radiation, even though ^{14}C-UBT is safe for children and pregnant women because radiation from ^{14}C-UBT is lower than radiation acquired from the natural environment. In the absence of expensive equipment and ability to pay high cost of ^{13}C-UBT, however, ^{14}C-UBT is more popular in the developing countries. The diagnostic accuracy between ^{13}C-UBT and ^{14}C-UBT is not different and both tests can be considered to be gold standard among the various noninvasive tests for the diagnosis of *H. pylori* infection[77]. There are two protocols, nonencapsulated and encapsulated, used for the oral administration of ^{14}C-urea to patients for *H. pylori* diagnosis. Initially, encapsulated ^{14}C-UBT was developed to avoid the problem of ^{14}C-urea hydrolysis by the action of urease-producing oral flora and this method obviated the problem of false-positive results in early breath samples[78]. Nonetheless, rapid transit of the ^{14}C-urea containing capsule from the gastric tract or its incomplete resolution in the stomach during the phase of breath collection causes encapsulated ^{14}C-UBT may not be a superior option than nonencapsulated protocol[79]. A recent study used dynamic scintiscan technique to monitor gastric fate of capsule and compared the sensitivity between nonencapsulated and encapsulated protocol in 100 dyspeptic patients. This study showed nonencapsulated protocol had higher sensitivity than encapsulated protocol and the sensitivity of encapsulated and nonencapsulated ^{14}C-UBT were 90.5% and 98.6% at 10 min and 91.8% and 97.2% at 15 min respectively. Incomplete or non-resolution of ^{14}C-urea capsule in stomach during the phase of breath collections noted by dynamic scintiscan images provided the explanation of lower sensitivity of encapsulated ^{14}C-UBT as compared with nonencapsulated ^{14}C-UBT[80].

The precise cut-off value for delta over baseline (DOB) value to discriminate between *H. pylori*-positive and *H. pylori*-negative results is the other controversial issue. The cut-off valve for the UBT was originally determined as 5.0‰, which had most widely recommended, whereas lower values, 3.0 or 3.5‰ were also proposed to improve its accuracy without compromising the sensitivity and specificity of this test. A "grey zone" in which the results of UBT are inconclusive were mentioned by previous studies and a borderline DOB value, like very close to the selected cut-off point, should be cautiously interpreted[81]. A novel method of UBT using an optical cavity-enhanced integrated cavity output spectroscopy system was introduced recently to provide optimal diagnostic cut-off point. This preliminary test defined diagnostic cut-off point as cumulative percentage of ^{13}C dose recovered (c-PDR) = 1.47% at 60 min and exhibited 100% sensitivity and 100% specificity with an accuracy of 100% as compared with invasive endoscopic tests. However, small number of samples are used in this study and further larger study is necessary to confirm these results[82].

STOOL ANTIGEN TEST

Stool antigen test (SAT) is the other noninvasive method with good sensitivity and specificity, 94% and 97% respectively in global meta-analysis, in the diagnosis of *H. pylori* infection[83]. This method detects the presence of *H. pylori* antigen in stool samples. There are two types of SATs used for H. pylori detection, enzyme immunoassay (EIA) and immunochromatography assay (ICA) based methods, using either polyclonal antibodies or monoclonal antibodies. Many SATs are available now for the diagnosis of *H. pylori* infection and different diagnostic accuracy are showed from different studies with different SATs and different study design. In general, monoclonal antibody-based tests are more accurate than polyclonal antibody-based tests[83] and EIA-based tests provide more reliable results than ICA-based tests[84,85]. In a recent study, the Tesmate pylori antigen (TPAg) EIA utilizing a monoclonal antibody to check native *H. pylori* catalase showed 92.4% sensitivity and 100% specificity in adult when compared with RT-PCR and the accuracy of this test was 94.9%[86]. Premier Platinum HpSA Plus test, the other monoclonal EIA-

based test, also showed reliable diagnostic results with 92.2% sensitivity, 94.4% specificity and 93.4% accuracy for diagnosing *H. pylori* infection as compared with the other 4 SATs, including 1 monoclonal EIA-based (*H. pylori* antigen test), 2 monoclonal ICA-based (ImmunoCard STAT! HpSA test and *H. pylori* fecal antigen test) and 1 polyclonal ICA-based (one-step *H. pylori* antigen test) tests, of which the accuracy were all lower than 90%[84]. However, ICA-based tests are easy to perform and do not require specialized equipment, which make it suitable for in-office test and developing countries. A new monoclonal ICA-based SAT, Atlas *H. pylori* Antigen Test, was also introduced recently and provide better results than previous monoclonal ICA-based SATs, with 91.7% sensitivity, 100% specificity and 96.6% accuracy[87].

As well as UBT, monoclonal EIA-based SAT is also a reliable test recommended by guidelines to assess the efficacy of *H. pylori* eradication therapy and the time for testing after the end of treatment should be as least 4 wk[21,88]. In previous meta-analysis, the pooled sensitivity and specificity for monoclonal SAT to confirm eradication after therapy were 93% and 96% respectively[83]. In recent studies, monoclonal EIA-based SATs have been confirmed to be a useful and accurate tool to determine the results of *H. pylori* eradication therapy, with 91.6%-100% sensitivity and 93.6%-98.4% specificity[89,90]. Furthermore, monoclonal ICA-based SATs, RAPID Hp StAR and ImmunoCard STAT! HpSA, also provide promising results with 90.0%-100% sensitivity and 93.6%-94.9% specificity.

In addition to assessment of eradication therapy, monoclonal SAT is a convenient, noninvasive and useful test for the diagnosis of *H. pylori* infection in pediatric patients[91]. A study applied SAT in children aged between 6 to 30 mo showed reliable results of SAT for diagnosing *H. pylori* infection in very young children[92]. A recent meta-analysis, including 45 studies and 5931 patients, to evaluate the performance of SATS in children showed pooled sensitivity and specificity were 92.1% and 94.1% respectively. In subgroup analysis, the sensitivity and specificity of monoclonal SAT, polyclonal SAT and one-step rapid monoclonal SAT were 96.2% and 94.7%, 88.0% and 93.0%, and 88.1% and 94.2% respectively. Monoclonal SAT is a reliable test for diagnosis of *H. pylori* infection in children[93]. Moreover, SAT is a useful tool for epidemiological study and screening programs[94,95]. With regard to cost and equipment, SAT is more suitable than UBT for mass surveys. As compared with serological test, which are usually used for screening, SAT seems to provide more reliable results in diagnosis of *H. pylori* infection. However, a previous study found SAT was less accurate than serological test in patients with severe atrophic gastritis and the influence of this result need further evaluation to assess the role of SAT in screening *H. pylori*-associated diseases, like gastric cancer[96]. Whereas the other study using a new polyclonal EIA-based SAT (EZ-STEP *H. pylori*) found presence of atrophic gastritis and/or intestinal metaplasia did not significantly affect the results of SAT[97].

The accuracy of SAT is influenced by several factors, like antibiotic, PPI, N-acetylcysteine, bowel movement and upper gastrointestinal bleeding. Preservation of the specimen, like temperature and transport time before testing, and cut-off valve also have impacts on the diagnostic accuarcy of SAT[98-100].

ANTIBODY-BASED TESTS

Numerous serological tests based on the detection of anti-*H. pylori* IgG antibody are widely available for *H. pylori* diagnosis and EIA test is the most common and accurate technique among them. Serological tests have also frequently been used in screening for epidemiological studies because of their inexpensive, rapid and acceptability to patients. Moreover, serological test is useful for evaluation of *H. pylori* infection in children. A recent study using E-Plate, a commercial serum antibody kit, to compare the performance of serological test with SAT in 73 children showed that the sensitivity, specificity, and positive likelihood ratio for serological test were 91.2%, 97.4%, and 35.6%, respectively. These results came from using recommended adult cutoff valve on children[101]. Because the accuracy of serological tests depends on the antigen used in commercial kit and the prevalence rate of specific *H. pylori* strains employed as the source of antigen. Proper antigens, either using local strains as the source of antigen or pooling antigens from strains of different groups, as well as reliable cutoff value of serological test should be validated locally before investigating population[102,103]. Several immunogenic proteins, like CagA, VacA, UreA, Omp and GroEL, have been used as candidates to detect infection. The *H. pylori* FliD protein, an essential element in the assembly of the functional flagella, is also recognized as a novel marker for serological diagnosis of *H. pylori* infection, with sensitivity and specificity of 99% and 97% respectively[104]. A novel line immunoassay, recomLine *H. pylori* IgG, which using six highly immunogenic virulence factors (CagA, VacA, GroEL, gGT, HcpC, and UreA) was introduced recently for serological diagnosis of *H. pylori* infection. The recomLine, in contrast to EIA and immunoblot, allows the identification of specific antibody response against distinct *H. pylori* antigens and increased discriminatory power. As compared to histology, the recomLine showed sensitivity and specificity of 97.6% and 96.2% respectively. The recomLine is also a useful tool to identify specific virulence factors of *H. pylori*[105,106].

The other advantage of serological test is that the accuracy of serological tests is not affected by ulcer bleeding, gastric atrophy as well as the use of PPI or antibiotics, which cause false negative results in other

invasive or noninvasive tests. However, serological test is not a reliable test to assess eradication therapy because antibody levels can persist in the blood for long periods of time even after successful eradication[21]. Because the serological tests do not distinguish between active infection and past exposure to *H. pylori*, further confirmation by other tests is required before eradication therapy.

Like SAT, EIA-based serological tests have better accuracy than ICA-based tests. A recent study comparing 29 commercial serological test (17 EIA-based and 12 ICA-based) showed the accuracy of 9 of 17 EIA-based tests were higher than 90%, whereas only one of the 12 ICA based tests had an accuracy > 90%. Heterogeneous performances were also observed between different serological tests, revealing sensitivity ranged from 57.8% to 100% and specificity ranged from 58.7% to 96.8% in EIA-based tests; sensitivity ranged from 55.6% to 97.8% and specificity ranged from 60.3% to 96.8% in ICA-based tests. The serological tests should be chosen properly according to their specific performance parameters to achieve different goals, like screening, initial diagnosis or confirmation of another test[107].

Serological test also play an important role in studies of pathogenesis and virulence factors because several antigenic proteins can be detected by immunological techniques and provide additional diagnostic value. Several attempts have been made to find potential biomarkers to identify patient infected with high-risk *H. pylori* strains by serological tests. Levels of pepsinogen (PG) Ⅰ, PG Ⅱ and PG Ⅰ/Ⅱ ratio combined with *H. pylori* antibody have been widely used to predict atrophic gastritis and risk of gastric cancer[108,109]. PG Ⅰ/Ⅱ ratio can also be useful in gastric cancer surveillance in patients after eradication therapy[110]. However, controversial results are presented on the clinical application of these serological makers. A recent study evaluating the accuracy of GastroPanel, which measures gastrin-17, *H. pylori* antibody, PG Ⅰ and PG Ⅱ, to detect atrophic gastritis showed only 50% sensitivity and 80% specificity, which were inferior to previous studies[111]. Pepsinogen test was also not accurate enough for the diagnosis of gastric cancer, with 71.0% sensitivity and 69.2% specificity[112]. Some virulence factors have also been evaluated to predict the prognosis of *H. pylori*-associated diseases. Presences of serum CagA, VacA, and GroEL antibodies in patients with *H. pylori* infection are associated with gastric precancerous lesions as well as gastric cancer and these serum markers might serve as potential predictors for patients infected with high-risk strains, which may be related to the development of gastric cancer[106,113]. Although the association between virulence factors and clinical presentations had been found by previous epidemiological studies, serological tests are still not reliable enough for diagnosis of gastric cancer. In a recent meta-analysis, the pooled sensitivity and specificity of CagA antibody using to diagnose gastric cancer were 71% and 40% respectively, and the diagnostic odds ratio were 2.11[114].

Detection of *H. pylori* IgG in urine had also been evaluated in children in previous studies, however, variable results were presented[115,116]. In addition, the diagnostic accuracy of EIA-based test to detect salivary *H. pylori* IgG was also not good enough as a reliable test[117,118]. Antibody detection in urine or saliva is less accurate than other tests and is not suggested to be used in the management of patients[119].

DIAGNOSIS OF *H. PYLORI* IN OTHER SPECIMENS

Utilizing PCR to detect *H. pylori* in stool is a reliable and rapid technique, which is especially attractive for children as a noninvasive test. Stool PCR also provides the advantages of identifying specific genotypes and antibiotic-resistance of the microorganism[120,121]. Oral cavity has been implicated as an extra-gastric reservoir of *H. pylori*, even though the significance of *H. pylori* in oral cavity, either a source of re-infection or the route of transmission, is still unclear. Saliva and dental plaque were the specimens commonly used to detect *H. pylori* in oral cavity and PCR was the most common and reliable test used in recent studies. RUT and culture were also performed to detect oral *H. pylori* in early studies. The prevalence of *H. pylori* detection in oral cavity exhibited wide variations, from 0% to 100%, and lower prevalence in saliva as compared with dental plaque was usually found[122]. The wide variations in the prevalence of *H. pylori* in oral cavity may be due to different methodologies, different populations and different primers used in studies. Recent studies focused on modification of primer to increase diagnostic accuracy or evaluation of new method to overcome the limitation of PCR. A novel PCR system, using a *H. pylori*-specific primer sets based on highly conserved sequences for the complete genomes of 48 *H. pylori* strains, was developed recently to increase the diagnostic accuracy of PCR in oral cavity[123]. The Loop-mediated Isothermal Amplification (LAMP), a new method of highly specific and sensitive DNA amplification, was compared with PCR on the detection rate of *H. pylori* in dental plaque samples in a small study which enrolled 45 participants. This study showed LAMP had higher detection rate than PCR and the detection rate of *H. pylori* in dental plaque samples by LAMP and PCR were 66.67% and 44% respectively[124].

DIAGNOSIS OF *H. PYLORI* IN SPECIFIC CLINICAL CIRCUMSTANCES

As mentioned previously, upper gastrointestinal bleeding (UGIB) decreases the diagnostic accuracy of many tests, including invasive and noninvasive, to

detect *H. pylori* infection. In a previous meta-analysis, RUT, histology and culture had low sensitivity and high specificity in patients with UGIB. UBT was still a reliable test, whereas SAT became less accurate in this clinical setting. Although serology was not influenced by UGIB, it could not be recommended as the first diagnostic test for *H. pylori* infection[40]. When comparing CLO, culture and histology, histology was less influenced by ulcer bleeding and could be a reliable test even in the presence of blood[125]. PCR had a significantly higher sensitivity than RUT, histology and culture, with sensitivity of 91%, 66%, 43% and 37% respectively and showed similar sensitivity as compared with serology and UBT, 94% and 94% respectively. PCR was similar to UBT in diagnostic accuracy for detecting *H. pylori* infection in bleeding peptic ulcers. However the specificity of PCR (100%) was only superior to serology (65%) and did not differ from other tests (RUT: 95%, histology: 95%, culture: 100%, UBT: 85%)[126]. A study also found RT-PCR could detection *H. pylori* infection by using formalin-fixed paraffin-embedded biopsy specimens in which histology showed negative results in patients with peptic ulcer bleeding[127]. Eradication of *H. pylori* is important in the management of *H. pylori*-associated ulcer bleeding for the purpose of preventing further bleeding and successful eradication therapy is even more effective than long-term maintenance antisecretory therapy with PPI to reduce rebleeding. Biopsy-based *H. pylori* testing is usually recommended during endoscopic survey of UGIB, even though bleeding decreases the sensitivity of biopsy-based tests. From the results of a meta-regression study, a delayed test, 4 wk after the UGIB episode, had higher detection rate of *H. pylori* in patients with UGIB. Because accurate determination of the etiology of bleeding ulcers is crucial in the management of ulcer bleeding, confirmation of a negative result with a subsequent noninvasive test has also been recommended by guidelines[22,128,129]. A low negative predictive value was also found when UBT was performed right after emergent endoscopy and a delay test was also mandatory for all negative results of early UBT[75]. Despite the importance of *H. pylori* testing in patients with UGIB, the proportion of patients who received direct *H. pylori* testing was quit low, about 12%-60% noted from previous studies. Concerns about decreased sensitivity related to bleeding or PPI use and increased risk of adverse events associated with gastric biopsies or increased procedure time to perform gastric biopsies may influence the decisions of *H. pylori* testing by clinicians[130].

Diagnosis of *H. pylori* in patients with partial gastrectomy is the other issue, although, to which less attention has been paid because these patients represented a very small portion of general population. In a meta-analysis comparing three commonly used tests in patients with partial gastrectomy showed histology performed the best, followed by the RUT, whereas the UBT had the poor diagnostic accuracy. These studies showed a high degree of heterogeneity and the pooled sensitivity and specificity of histology, RUT, and UBT were 93% and 85%; 79% and 94%; 77% and 89% respectively. The RUT was suggested as the initial choice of test on these patients and biopsy samples from gastric fundus or the upper body of the remnant stomach was recommended. Histology was recommended to performed after negative result of RUT in these patients[131]. SAT may be the other reliable test to detect *H. pylori* in patients with distal gastrectomy. A small study using HpSA test to evaluate the diagnostic accuracy of SAT in 59 patients with distal gastrectomy for gastric cancer demonstrated that the sensitivity, specificity and accuracy of HpSA test were 100%, 90.5%, and 96.6%, respectively[132]. The possible reason for inadequate performance of UBT in the diagnosis of *H. pylori* in patients with distal gastrectomy may be not enough time for the urea stays in the gastric stump to interact with urease produced by *H. pylori.* The BreathID, a rapid continuous-real-time UBT, seemed to overcome this shortcoming and it showed better accuracy than RUT, 87% and 72% respectively. However lower sensitivity and specificity of RUT, 82% and 71% respectively, as compared with previous studies was also found in this study and biopsies were taken from the gastric body slightly distal to fundus in this study may influenced the diagnostic performances of RUT[133]. A recent study also demonstrated discordant results between UBT and biopsy-based tests in patients with partial gastectomy after *H. pylori* eradication therapy. The authors suggested additional endoscopic biopsy-based tests would be helpful to avoid unnecessary treatment because high false positive rate and low positive predictive value of UBT, 19.1% and 44.7% respectively, were found in these patients after eradication therapy[134].

Accurate determination of *H. pylori* status in patients after eradication therapy is important and UBT as well as SAT are recommended by guidelines to assess the efficacy of eradication therapy. These tests are usually recommended to perform more than 4 wk after end of therapy[21,88]. However, high false positive rate of 52.9% was found by using ^{13}C-UBT with current cutoff value (2.5‰), especially in patients with more than two times previous eradication therapies and in patients with moderate to severe gastric intestinal metaplasia[135]. A recent study using nested PCR to detect *H. pylori* from gastric biopsy specimens after eradication therapy showed nested PCR is more sensitive than RUT, histology and culture. Furthermore, PCR based method is able to discriminate the reinfection or recrudescence after eradication therapy[136].

CONCLUSION

The developments of current diagnostic methods

allow to have a more accurate diagnosis of *H. pylori* infection, which in turn improving the management of *H. pylori*-associated diseases. Although the golden standard test may not exist, the choice of test to detect *H. pylori* infection depends on the prevalence and strains of *H. pylori* on endemic areas, accessibility, advantages and disadvantages of each method as well as different clinical circumstances of each patient. To combine the results of two or more tests could be a reasonable strategy in routine clinical practice to achieve the most reliable result. We believe that there will be continuous attempts to evolve the diagnostic yield of *H. pylori* infection for different clinical purposes, specific populations, and genotypic characterizations to have more reliable and feasible diagnostic modalities of *H. pylori* infection in the future.

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TOPIC HIGHLIGHT

2015 Advances in Inflammatory Bowel Disease

Promises and paradoxes of regulatory T cells in inflammatory bowel disease

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Abstract

Since their discovery two decades ago, $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs) have become the subject of intense investigation by immunologists. Unlike other T cells, which promote an immune response, Tregs actively inhibit inflammation when activated by their cognate antigen, thus raising hope that these cells could be engineered into a highly targeted, antigen-specific, immunosuppressant therapy. Although Tregs represent less than 10% of circulating $CD4^+$T cells, they have been shown to play an essential role in preventing or limiting inflammation in a variety of animal models and human diseases. In particular, spontaneous intestinal inflammation has been shown to occur in the absence of Tregs, suggesting that there may be a Treg defect central to the pathogenesis of human inflammatory bowel disease (IBD). However, over the past decade, multiple groups have reported no qualitative or quantitative deficits in Tregs from the intestines and blood of IBD patients to explain why these cells fail to regulate inflammation in Crohn's disease and ulcerative colitis. In this review, we will discuss the history of Tregs, what is known about them in IBD, and what progress and obstacles have been seen with efforts to employ them for therapeutic benefit.

Key words: Foxp3; Regulatory T cells; Crohn's disease; Th17; Ulcerative colitis; Inflammatory bowel disease

Core tip: Regulatory T cells (Tregs) have received much interest in animal models of inflammatory bowel disease (IBD), but have yet to demonstrate a clear defect in human Crohn's disease or ulcerative colitis. This review will detail our current knowledge about this important regulatory arm of the immune system in human IBD, and discuss the potential role for Tregs as immunotherapy.

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INTRODUCTION

In the summer of 1995, Shimon Sakaguchi published the first report of what later came to be recognized as regulatory T cells (Tregs) by demonstrating that mice depleted of $CD4^+CD25^+$ T cells spontaneously developed multiorgan autoimmunity, including gastrointestinal (GI) inflammation[1]. More importantly, such autoimmunity could be prevented by administration of these $CD4^+CD25^+$ Tregs, suggesting that they might someday represent a potent cellular therapy for autoimmune and chronic inflammatory conditions. In the twenty years since this initial report, well over 10000 original manuscripts have been published concerning Tregs, making them one of the most intensely studied T cell populations of the 21st century.

Interest in Tregs took a quantum leap forward shortly after the turn of the millennium, when it was discovered that the gene *FOXP3* was central to Treg development and function, and could serve as an excellent marker for these relatively rare cells. A genetic defect in the *FOXP3* gene which precluded Treg development was found to be the cause of a mouse multiorgan inflammatory condition called scurfy[2]. At roughly the same time, a similar human condition called immune polyendocrinopathy enteropathy X-linked (IPEX) was reported to result from mutations in the human *FOXP3* gene resulting in humans with no Tregs[3,4]. As the name implies, an inflammatory enteropathy, resembling severe pan-intestinal Crohn's disease, is a central feature of IPEX, and generally causes fatal malnutrition in the absence of a hematopoietic cell transplant (HCT).This condition made it clear that the Tregs which had been receiving increasing attention in murine models were also critical for intestinal immune homeostasis in humans.

TREG MECHANISMAS OF ACTION

We now know that $FOXP3^+$ Tregs reside within the intestinal lamina propria and represent up to 10% of circulating $CD4^+$ T cells in humans[5-8]. Tregs recognize specific MHC-II-bound peptide antigens though a clonally unique T cell receptor (TCR), just like any other $CD4^+$ T cells[7,9]. However, while other T cells will deliver pro-inflammatory signals upon TCR ligation, Tregs do the opposite. They inhibit the activation of bystander T cells in a contact-dependent manner[10]. While no single molecular mechanism for this inhibition has been elucidated, several regulatory signals appear to be important (Figure 1), augmentation of which would represent an attractive opportunity for IBD therapy.

By definition, Tregs express more CD25 than any other T cells[1], and because CD25 is an essential component of the high-affinity IL-2 receptor, Tregs may absorb local IL-2, depriving nearby T cells of this T cell growth and survival factor when its concentration is limiting. However, IL-2 is evidently not essential for pro-inflammatory T cell growth and survival because mice genetically engineered to lack CD25[11] or the beta chain of the IL-2 receptor (CD122)[12] do not develop immunodeficiency, but rather a lymphoproliferative disorder including spontaneous autoimmunity and IBD. This was evidently due to a lack of Tregs[13], as the latter are uniquely dependent upon IL-2. Thus, depriving other T cells of IL-2 is certainly not central to the inhibitory effect of Tregs *in vivo*.

Tregs also constitutively express more of the immunoregulatory CTLA4 molecule (CD152) than other T cells[8,14,15], and this molecule appears to be necessary for Treg inhibitory function[15,16]. CTLA4 can bind up B7-1 (CD80) and B7-2 (CD86) costimulatory molecules on the surface of antigen presenting cells (APC), preventing them from costimulating CD28 receptors on other T cells[17]. Mice lacking the CTLA4 gene develop multiorgan autoimmunity[18] not unlike mice lacking Tregs. Similarly, patients who receive the CTLA4-blocking antibody ipilimumab as a cancer immunotherapy can develop spontaneous autoimmunity, including enterocolitis in over 20% of recipients[19,20], thus demonstrating the importance of this molecule in maintaining intestinal immune homeostasis. However, whether CTLA4's role is primarily mediated through Tregs is unclear, as ipilimumab also limits CTLA4 engagement on activated T cells.

TIGIT, a molecule analogous to CTLA4, is also enriched on a subset of Tregs[21,22], and likewise binds costimulatory molecules (CD112, CD155) on APC, preventing them from ligating a costimulatory receptor (CD226) on effector T cells, and thereby inhibiting the latter[23]. $TIGIT^+$ Tregs have been reported to selectively inhibit Th1 and Th17 cells, the $CD4^+$ T cell populations commonly associated with autoimmune and inflammatory conditions like IBD[24]. Tregs also express PD-1 (CD279)[25], an inhibitory receptor that interacts with PD-L1 (CD274) and PD-L2 (B7-DC, CD273) on APCs and has, like CTLA4, recently become a target for cancer immunotherapy[26-29]. Like CTLA4 blockade, PD-1 blockade has caused spontaneous intestinal inflammation in clinical trials, albeit at a lower rate, affecting < 10% of recipients[30,31].

In addition to their contact-dependent immunomodulatory mechanisms, Tregs may control inflammation through soluble factors. CD39 is an ectonucleotidase preferentially expressed by Tregs, which hydrolizes ATP and ADP to AMP, and ultimately adenosine[32,33]. ATP has been reported to enhance pro-inflammatory Th17 cells[34,35], while adenosine may inhibit effector T cells through the A_{2A} receptor[36-39], so this surface receptor may change the local environment of the Tregs to regulate inflammation.

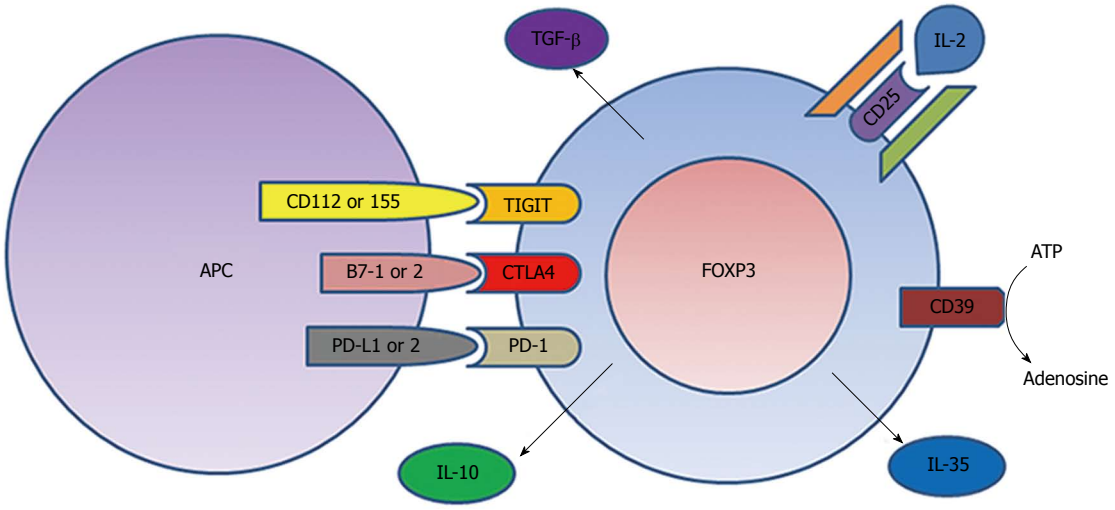

Figure 1 FOXP3$^+$ Tregs may mediate their inhibitory function through multiple soluble and cell-surface factors. CTLA4, TIGIT and PD-1 interact with costimulatory molecules on antigen presenting cells (APC). CD25 binds the T cell growth factor IL-2. CD39 converts local ATP to adenosine. The cytokines IL-10, IL-35 and TGF-β have suppressive functions on nearby immune cells.

Reduced Treg expression of CD39 has been described in lupus[40] and multiple sclerosis[32,32,41], but has not yet been described in IBD.

Tregs have also been reported to control inflammation through cytokines. TGF-β is expressed by Tregs, and has immunomodulatory properties, although it may function as a cell-surface protein on Tregs[42], and may not be necessary for Treg inhibitory function[43]. IL-10 is likewise an immunomodulatory cytokine made by Tregs[42], and is essential for preventing spontaneous bowel inflammation in mice[44] and humans[45]. However, the immunoregulatory roles of IL-10 and TGF-β may be more appropriately ascribed to other "regulatory T cell" populations that do not express FOXP3, namely Tr1[46,47] and Th3 cells[48], which are beyond the scope of this review. More recently, FOXP3$^+$ Tregs have been shown to mediate their inhibitory function through the cytokine IL-35[49,50].

TREGS IN IBD

A number of clinical observations and experiments in animal models[51,52] have suggested that Tregs or their inhibitory mechanisms are critical for preventing spontaneous intestinal inflammation, and thus suggested that a defect in Tregs may be central to the pathogenesis of UC and/or Crohn's disease. Out of 38 distinct animal models of IBD reviewed in 2003, nine involved Tregs or their inhibitory mechanisms[51,53]. As an iatrogenic inflammatory bowel disease, human gastrointestinal graft *vs* host disease (GVHD) following HCT has been associated with evidence of decreased Tregs in the blood[54] and intestinal mucosa[55].

Despite this wealth of data implicating Tregs in intestinal immune homeostasis, direct evaluation of Tregs in the intestines of IBD patients has not identified obvious defects. The first report of CD4$^+$CD25$^+$ Tregs isolated from the intestinal lamina propria (LP) of IBD patients, published more than a decade ago, demonstrated that these cells are present, express CTLA4, and show *in vitro* suppressive activity against other T cells which is no different from those of controls[56]. This and subsequent reports found that these Tregs paradoxically represent a greater fraction of LP CD4$^+$ T cells in the intestines of IBD patients than healthy control subjects[5] and are no less common in bowel affected by IBD than in bowel inflamed for other reasons, such as infection[51]. Paradoxically, Tregs are even more common in actively inflamed than uninflamed IBD mucosa[5,57-59], with a reciprocal drop in circulating Treg frequency in the peripheral blood of symptomatic IBD patients likely reflecting sequestration of these cells to the site of inflammation. Thus, the mucosal inflammation of IBD appears to be different from that of IPEX in that it does not result from any local dearth of FOXP3$^+$ cells.

ACTIVATION INDUCED FOXP3 EXPRESSION

Confounding these analyses was the discovery that FOXP3 expression could be induced *de novo* in human T cells that were originally FOXP3 negative by TCR activation in the presence of TGF-β[60,61]. Thus the seemingly paradoxical excess of FOXP3$^+$ cells in the inflamed mucosa of an IBD patient could simply be locally activated T cells. Complicating matters, by some accounts, T cells induced to express FOXP3 by activation are nonetheless effective regulators of other immune cells *in vitro*[62,63]. Whether these "induced Tregs" (iTregs) have all the same suppressive function *in vivo* as constitutively FOXP3$^+$ "natural" Tregs (nTregs) has been debated[64], and is difficult to establish experimentally in humans. One significant difference

between iTregs and nTregs concerns their ability to make cytokines. Classical nTregs do not make pro-inflammatory cytokines, such as IL-2 or IFN-γ, and additionally show demethylation of CpG sites in the FOXP3 promoter[6]. In contrast, iTregs generated from effector T cells retain their ability to produce these cytokines[64], and do not demethylate their FOXP3 promoter[65], although they do up-regulate CD25 and CTLA4 to resemble nTregs[64], making it difficult to discern the two Treg populations by surface markers. Adding to the complexity, it has become clear that the "nTregs" that constitutively express FOXP3 *in vivo* are actually a mix of Tregs that either acquired FOXP3 expression in the thymus (tTregs) or periphery (pTregs), thus reflecting their antigen specificity and perhaps phenotype[66].

The nuclear protein Helios has been shown to be constitutively expressed by thymically-derived tTregs, but not *in vitro*-generated iTregs[67], making this a potentially unique marker with which to distinguish at least these two populations. The fraction of $FOXP3^+$ LP T cells that express Helios is no lower in IBD patients than controls[68], suggesting that the paradoxically increased $FOXP3^+$ T cells in IBD are not exclusively iTregs. However, there is evidence that activation-induced $FOXP3^+$ T cells may acquire Helios expression[69], thus compromising the reliability of Helios as a marker for distinguishing iTregs from nTregs.

The TCR gene is uniquely rearranged in each nascent T cell, making it a stable genetic marker with which to identify T cells from a common clonal origin. By comparing the TCR Vβ hypervariable domain repertoires of $FOXP3^+$ and $FOXP3^-$ T cell populations from the colon LP, it has been shown that these are predominantly distinct populations, even in IBD[68]. Indeed, LP $Helios^-$ Tregs show no more similarity in their TCR repertoire to effector T cells than they do to $Helios^+$ Tregs[68]. Thus, the paradoxically increased mucosal $FOXP3^+$ cells in IBD cannot be explained solely by activation-induced FOXP3 expression among effector T cells.

TREG *VS* TH17 CELLS

Several groups have noted that an unusually high fraction of mucosal Tregs from IBD patients are able to produce IL-17A[70-72]. IL-17A is a potent pro-inflammatory cytokine associated with neutrophil recruitment[73], and hence thought to play a central role in anti-bacterial immune responses. It is made by a subset of effector T cells, called Th17 cells, which can be identified by CCR6[74] and CD161 expression[75], and have been implicated in multiple autoimmune conditions[76]. Thus, by sharing characteristics with a potentially pathogenic class of T cells, the copious intestinal $FOXP3^+$ Tregs present in IBD could paradoxically promote rather than suppress intestinal inflammation.

Like iTregs, Th17 cells require TGF-β for their development, but additionally require IL-6, which in turn suppresses the formation of $FOXP3^+$ Tregs[77,78]. The differentiation of Th17 cells is governed by the transcription factor RORγt[74,79] instead of FOXP3. In cells that express both transcription factors, FOXP3 physically interacts with RORγt in the nucleus to prevent the latter from promoting IL-17A expression[80]. This interaction requires a region of the FOXP3 protein encoded by exon 2 of the FOXP3 mRNA[80], which is deleted in a splice variant (Δexon 2) that represents approximately half the FOXP3 transcripts expressed by humans[81]. This would suggest that IL-17-producing $FOXP3^+$ T cells, as seen in IBD, could be exclusively expressing the Δexon 2 variant of FOXP3. However, no predominance of Δexon 2 relative to full-length FOXP3 expression is seen in IBD, nor are there cells which exclusively express Δexon 2, even among IL-17-expressing $FOXP3^+$ T cells[57]. Thus, how Th17-like $FOXP3^+$ T cells arise in IBD remains a mystery, but could be due to an increased responsiveness to IL-6, as has been seen in T cells from multiple sclerosis patients[82].

TREG AND THE INTESTINAL FLORA

With the recent advent of inexpensive, high-throughput nucleic acid sequencing techniques, the bacterial flora, or "microbiome", of the GI tract has recently come under intense scrutiny. Differences between the intestinal microbiomes of people with and without IBD have been described by many independent researchers[83-86], although it is difficult to determine whether such differences are a cause or effect of IBD once sufficient inflammation has occurred in the GI tract to diagnose an individual with IBD. Nonetheless, a leading hypothesis about the pathogenesis of IBD dictates that the immune system is losing tolerance to intestinal commensal flora, suggesting a dominant role for the microbiome.

Studies in germ-free mice have demonstrated that the gut microbiome is important for development of the normal intestinal immune system, as reviewed elsewhere[87]. This includes IL-10-producing, peripherally-induced $FOXP3^+$ Tregs, whose development can be driven by specific intestinal microbiota in animal models[88,89]. While some intestinal Treg development may simply be due to exposure to luminal peptide antigens, non-peptide bacterial products, such as short-chain fatty acids[90] or specific polysaccharides[88], are important for Treg induction in the gut. Likewise, ingested micronutrients, such as retinoic acid, have been shown to contribute to the peripheral generation of $FOXP3^+$ Tregs in the gut[91]. Thus, exposure of the intestinal mucosa to the fecal stream may be an important means by which the mucosal immune system develops tolerance, or perhaps fails to do so in IBD.

TREG IN IBD THERAPY

Contemporaneous with the growth of research on Tregs in the early 21st century was the use of biopharmaceutical therapy for IBD and other inflammatory conditions involving TNF-α blockade. Perhaps as a consequence, a number of groups analyzed the effect of anti-TNF agents, particularly infliximab, on circulating FOXP3$^+$ Tregs, and found that the latter were enriched in the peripheral blood of patients demonstrating a good clinical response to therapy[92-95]. This suggests that the blockade of TNF-α *in vivo* may enhance Treg development, expansion, or viability if this cytokine normally inhibits Tregs in the setting of inflammation. Alternatively, because anti-TNF drugs can cause apoptosis of TNF-producing cells, and Tregs do not make TNF-α, it is possible this effect reflects a selective "pruning" of the FOXP3- effector T cell population rather than expansion of FOXP3$^+$ Tregs. However, caution should be taken in drawing conclusions about IBD from peripheral blood analyses, as the intestinal lamina propria houses more lymphocytes than the circulation. Thus selective sequestration or release of cell populations to or from the gut can actually cause the blood to reflect the opposite of what is actually happening at the site of inflammation in IBD. Indeed, the effect of anti-TNF agents on intramucosal Tregs has been less clear, with some researchers reporting a drop in FOXP3$^+$ cells on therapy[94], and others reporting an increase[95]. Further confounding these analyses is the observation that histological IBD activity correlates inversely with Treg frequency in tissue sections[5,57-59], such that a drop in tissue Tregs in the setting of effective therapy could obscure any local enrichment, and if mediated by a release of Tregs into circulation, produce the observed increase in blood Tregs.

The effect of other immunosuppressive therapies on Tregs has been less intensely studied in IBD, but data exists from other conditions for which these drugs are used. In liver transplant recipients, use of the immunosuppressive drug azathioprine has been paradoxically associated with decreased colonic FOXP3$^+$ cells, although only as cotherapy with prednisone and calcineurin inhibitors[96]. Likewise, in autoimmune hepatitis, azathioprine use, again in conjunction with prednisone, resulted in decreased intrahepatic Tregs, although a higher ratio of these Tregs to other lymphocytes correlated with biochemical remission[97]. Although these effects could be attributed to cotherapy with prednisone, studies in asthmatics have shown no effect of oral glucocorticoids on circulating Treg frequency[97]. Furthermore, as with anti-TNF agents, it is difficult to demonstrate that changes in Tregs associated with a given therapy represent a cause or effect of changes in inflammatory activity. Whether the newer anti-integrin biopharmaceutical vedolizumab will have an effect on intramucosal Tregs has yet to be seen, but a similar agent, natalizumab, did not alter the ratio of Tregs to other T cells in the intestinal mucosa of Crohn's patients receiving it[98].

TREGS AS IBD THERAPY

Shortly after their discovery, Tregs were proposed as a potential therapy for autoimmune or inflammatory disease in more reviews and editorials than can be listed here. Indeed, in many animal models, adoptive transfer of Tregs proved effective for the prevention or treatment of inflammatory conditions, including IBD[99]. However, more than a decade later, the application of Tregs to human disease has been surprisingly limited. Given their rarity in peripheral blood, a major obstacle to therapeutic application of Tregs has been simply having enough Tregs to administer, so much work went into expanding or generating Tregs *in vitro* into a large, stable population with stable suppressive function. The earliest and most extensive efforts applying Tregs as anti-inflammatory therapy have been directed at GVHD complicating HCT[100-102], a condition which, like IBD, commonly involves deregulated intestinal inflammation. As an alternative to adoptive transfer of *in vitro* expanded Tregs, *in vivo* expansion of Tregs post HCT through the use of low-dose IL-2 has demonstrated efficacy against GVHD[103-105]. Low dose IL-2 also expanded Tregs in type- Ⅰ diabetes[106,107], but it paradoxically accelerated autoimmunity, even when given with the immunosuppressant rapamycin, perhaps because it also expanded eosinophils and NK cells[107]. However, some efficacy has been seen with adoptive transfer of Tregs in type- Ⅰ diabetes[108,109].

The first trial of adoptive transfer of Tregs as a therapy for IBD was recently published as an 8-wk, open-label, dose-ranging study involving 20 Crohn's patients[110]. In contrast to the aforementioned trials in GVHD and diabetes, the transferred Tregs were selected and cloned to be specific for a dietary antigen (chicken egg ovalbumin) so that antigen-specific activation of the transferred cells could be stimulated in the gastrointestinal tract through an egg-intensive diet (meringue cake). 40% of recipients demonstrated clinical improvement, although the most improvement was paradoxically seen in recipients of the smallest number of Tregs (10^6), and only minimal improvement was observed by objective measures of inflammation, such as C reactive protein and fecal calprotectin. Thus, the efficacy of Tregs as IBD therapy was neither straightforward nor overwhelming, suggesting that other factors, such as Treg antigen specificity or inhibitory function, may be more important than Treg numbers. Curiously, the number of circulating FOXP3$^+$ T cells decreased in responders, while rising in non-responders. However, the frequency of Tregs in the intestines was not evaluated, so this dichotomy could reflect mucosal Treg sequestration if such a phenomenon was associated with therapeutic response.

CONCLUSION

Despite extensive interest in Tregs as central mediators of intestinal immune homeostasis, there is surprisingly little evidence that a defect in Tregs is associated with either form of human IBD. The fact that inflammation persists in Crohn's and UC despite an excess of Tregs in the mucosa relative to healthy bowel indicates that the inflammation of IBD is resistant to their presence. Whether the mucosal Tregs of IBD patients are intrinsically defective in their ability to regulate mucosal inflammation *in vivo* is unknown, but *in vitro* assays have shown no such functional defect[56,58,59]. Alternatively, Treg-extrinsic factors could undermine the immunoregulatory function of Tregs. Other immune cells, such as FOXP3-negative effector T cells, could be resistant to the inhibitory function of Tregs in IBD, as has been described in multiple sclerosis and diabetes[82,111]. Mucosal dendritic and other antigen presenting cells with which Tregs and other T cells interact could deliver signals which undermine Treg-mediated inhibition. Finally, the mucosal micro-environment in general, including soluble factors and components of the extracellular matrix, such as hyaluronic acid[112], could be actively detrimental to, or passively unsupportive of, the inhibitory function of Tregs in IBD. A better understanding of the factors that undermine Treg function in IBD will be necessary before the promise of Tregs as an IBD therapy can ultimately be realized.

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TOPIC HIGHLIGHT

2015 Advances in Inflammatory Bowel Disease

Disease monitoring in inflammatory bowel disease

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Abstract

The optimal method for monitoring quiescent disease in patients with Crohn's disease (CD) and ulcerative colitis is yet to be determined. Endoscopic evaluation with ileocolonoscopy is the gold standard but is invasive, costly, and time-consuming. There are many commercially available biomarkers that may be used in clinical practice to evaluate disease status in patients with inflammatory bowel disease (IBD), but the most widely adopted biomarkers are C-reactive protein (CRP) and fecal calprotectin (FC). This review summarizes the evidence for utilizing CRP and FC for monitoring IBD during clinical remission and after surgical resection. Endoscopic correlation with CRP and FC is evaluated in each disease state. Advantages and drawbacks of each biomarker are discussed with special consideration of isolated ileal CD. Fecal immunochemical testing, traditionally used for colorectal cancer screening, is mentioned as a potential new alternative assay in the evaluation of IBD. Based on a mixture of information gleaned from biomarkers, clinical status, and endoscopic evaluation, the best treatment decisions can be made for the patient with IBD.

Key words: Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Fecal calprotectin; C-reactive protein; Fecal immunochemical test; Biomarkers; Remission; Postoperative recurrence

Core tip: C-reactive protein (CRP) is not specific for intestinal inflammation but does have modest correlation with clinical and endoscopic findings in inflammatory bowel disease patients. CRP can be falsely low despite active mucosal inflammation and is more reliable in cases of transmural inflammation. Fecal calprotectin (FC) is more specific than CRP for intestinal inflammation, except in isolated ileal disease. FC better correlates with endoscopic findings than CRP and is useful in monitoring Crohn's patients for postoperative recurrence. Optimal FC cutoffs are still being determined.

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INTRODUCTION

The clinical course of inflammatory bowel disease (IBD) varies widely from patient to patient. Whereas some patients are able to stay in remission for years with minimal treatment, other patients have a chronic, relapsing course with frequent flares despite aggressive therapy[1]. Twenty percent of Crohn's patients will relapse yearly, and 67% of Crohn's patients cycle between relapse and remission in the first 8 years after diagnosis. In ulcerative colitis (UC), there is a 9% to 21% 10-year cumulative risk of colectomy[2]. Given the known risk of disease progression in IBD, it is important to monitor for active disease and optimize treatment plans accordingly.

In the past, physicians have focused on clinical symptoms and clinical remission to guide treatment. However, it has been established that a patient's clinical symptoms, particularly with Crohn's disease (CD), are frequently inconsistent with endoscopic findings[3]. More recently, the goal of mucosal healing has emerged as the new treatment target[4]. In multiple trials, mucosal healing has been shown to improve long-term outcomes such as avoidance of surgery and fewer hospitalizations[5-7]. While endoscopic evaluation is the gold standard for assessment of mucosal inflammation, less invasive and less time-consuming modalities for assessing inflammation are valuable in day-to-day management.

Relapses are often difficult to predict. The goal of disease monitoring is to identify patients at risk for relapse in order to treat earlier, with the hope of maintaining remission and avoiding irreversible bowel damage such as fistulas and strictures that may lead to surgery.

The optimal method for monitoring disease activity in CD and UC is still being defined. Current modalities for assessing disease activity include colonoscopy, clinical assessment tools, serum biomarkers, fecal biomarkers, and imaging examinations such as CT enterography, small bowel follow-through, and MR enterography.

Many quantifiable laboratory assessments have been studied for evaluation of disease activity in IBD. Examples of commonly available serum lab assays include C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), leukocytes, platelets, ferritin, haptoglobin, ceruloplasmin, α-1-antitrypsin, plasminogen, complement factors, and fibrinogen[8]. More experimental serum assays that are not widely commercially available include orosomucoid (α-1-acid glycoprotein), interleukin 6 (IL-6), sialic acid, and serum amyloid A. Stool assays for detecting inflammation include fecal calprotectin (FC), lactoferrin, polymorphonuclear elastase, myeloperoxidase, metalloproteinase-9, and neopterin. MicroRNA species[9] and proteomic profiles[10], available only in research settings, have also been shown to differentiate active *vs* inactive IBD.

Of these diverse assays, CRP and FC are the most widely adopted in clinical practice for disease monitoring in IBD. This is a review of the current medical literature regarding the use of these two commonly utilized biomarkers for monitoring of disease to predict relapse in patients in clinical remission and in the postoperative setting.

CRP

C-reactive protein was first described in 1930 by Tillet and Francis[11]. Patients with pneumonia were noted to have serum that precipitated when brought in contact with bacterial "Fraction C" substance in the supernatant. This precipitant was no longer present in serum after the pneumonia resolved but was persistently present in lethal cases.

CRP is a pentameric, acute-phase protein made by hepatocytes[12]. The half-life of CRP is 19 hours, which allows for rapid rising and falling of levels with onset of and resolution of inflammatory states, respectively. Healthy individuals have low levels of CRP in circulation, usually less than 1 mg/L, but levels can rise 100-fold in periods of acute inflammation[13].

CRP is not a specific marker for intestinal inflammation. Measurements of CRP may be elevated for other reasons such as infection or extraintestinal inflammation. CRP has been studied outside of gastroenterology to predict disease outcomes after myocardial infarction and diagnosis of multiple myeloma[14,15]. In IBD, CRP has been significantly associated with other biomarkers of inflammation including ESR, thrombocytosis, anemia, and hypoalbuminemia[16]. As a biomarker, CRP is appealing because it is inexpensive, minimally invasive, and quick to result.

CRP CORRELATION WITH ENDOSCOPY

CRP is often used to monitor for occult internal inflammation when patients are clinically asymptomatic. In general, CRP is more frequently elevated in active transmural CD than in mild to moderate mucosal inflammation associated with UC[17-20]. Though not always accurate or specific, clinical disease activity in adults and children with CD has been shown to correlate with CRP level[16,21,22]. However, 20%-25% of CD patients having flares do not exhibit increased CRP due to genetic single nucleotide polymorphisms in the CRP gene, which affects CRP production[23].

Several studies have reported good correlation

Table 1 Endoscopic score correlation with C-reactive protein

Ref.	Disease	Endoscopic tool	Correlation	*P* value
Sipponen *et al*[88]	CD	CDEIS	$r = 0.608$	< 0.001
Schoepfer *et al*[77]	CD	CDEIS	$r = 0.75$	< 0.010
af Björkesten *et al*[25]	CD	SES-CD	$r = 0.56$	< 0.001
Lobatón *et al*[48]	UC	Mayo	$r = 0.307$	< 0.001

CDEIS: Crohn's disease endoscopic index of severity; SES-CD: Simple endoscopic score for Crohn's disease; *r*: Spearman's rank correlation coefficient.

between CRP levels and findings seen during endoscopy[16,24,25] (Table 1). Solem *et al*[16] reported a retrospective cohort of 104 CD patients. CRP was found to be normal in 75% of the CD patients with normal ileocolonoscopy. On the other hand, CRP elevations were significantly associated with active mucosal inflammation on colonoscopy (OR = 3.5, 95%CI: 1.4-8.9) defined as erosions, ulcerations, spontaneous bleeding, exudate, friability, granularity, cobblestoning, extensive erythema, inflammatory-appearing nodularity, and masses. CRP elevations (> 0.8 mg/dL) were significantly associated with moderate to severe clinical activity (OR = 4.5, 95%CI: 1.1-18.3) as defined by ACG clinical practice guidelines[26] (Table 2). Notably, in this study, there was no significant correlation between abnormal small bowel imaging and CRP elevation, suggesting that CRP could be normal in patients with isolated small bowel CD, but there was no subgroup analysis of isolated endoscopic ileitis in relation to CRP.

Henriksen *et al*[27] studied CRP levels according to disease subtype in 176 Crohn's patients and 371 UC patients. For CD, there were no significant differences in CRP levels based on disease localization (ileitis, colitis, or ileocolitis), showing that isolated ileal disease also caused a rise in CRP. For both UC and CD, CRP responses increased based on extent of disease. However, the mean and median levels of CRP in UC were within the normal range for CRP (< 10 mg/L) for all disease subgroups, making CRP less informative in UC disease monitoring.

In a prospective study of 64 CD patients on anti-TNF therapy, endoscopic SES-CD activity score correlated better with CRP ($r = 0.56$, $P < 0.001$) than with clinical indices including the CD Activity Index (CDAI) ($r = 0.40$, $P < 0.001$) and the Harvey Bradshaw Index (HBI) ($r = 0.32$, $P < 0.001$)[25]. However, CRP was not reliable in predicting endoscopic remission; the CRP was falsely negative (< 3 mg/L) nearly twice as often as the SES-CD indicated endoscopic remission.

Mosli *et al*[28] completed a meta-analysis comprised of 19 studies ($n = 2499$ IBD patients) to characterize CRP correlation with endoscopic disease activity. For IBD, CRP levels had a pooled sensitivity and specificity of 49% and 92%, respectively. There were an insufficient number of studies to calculate separate CRP performance metrics for UC and CD. The authors suggested a CRP cutoff of greater than 5mg/dL to indicate active endoscopic disease.

PREDICTION OF RELAPSE USING CRP

High CRP levels correlate with clinical relapse in both short-term and long-term follow up[29-32]. Various studies have reported an increased risk of relapse with the relative risk ranging from 3 to 58[30-32]. In severe UC flares, high CRP, combined with high stool frequency and low serum albumin, has been associated with higher likelihood of failure to respond to medical therapy[33,34]. There is also a 6-times higher risk of hospitalization (OR = 6.82, 95%CI: 2.5-18.58; $P < 0.0001$) with elevated CRP in CD patients[35].

In analysis of the GETAID trial, 71 CD patients in medically-induced clinical remission had CRP, complete blood count, erythrocyte sedimentation rate (ESR), alpha-1 antritrypsin, and orosomucoid, checked every 6 wk[32]. Thirty-eight patients clinically relapsed, defined as a CDAI greater than 150 or increase of at least 100 points from baseline, after a median of 31 wk. Only ESR greater than 15 mm and CRP greater than 20 mg/L predicted clinical relapse. Levels of CRP were noted to rise 4 to 6 mo prior to clinical relapse, suggesting that routine measurement of biomarkers every 3-4 mo could alert the clinician that an alteration in therapy may be necessary.

Achieving not only clinical remission but also mucosal healing may lead to higher rates of long-term response or remission. In post-hoc analysis for the ACCENT-1 trial, 137 CD patients in clinical remission had CRP levels measured after induction with infliximab. At week 14, 56.6% of patients with a CRP less than 0.5 mg/dL *vs* 37.2% of patients with a CRP greater than 0.5 mg/dL maintained response to infliximab through 54 wk ($P = 0.005$)[36].

Rapid normalization of CRP levels correlates with sustained long-term response to infliximab[37] and adalimumab[24]. Jurgens *et al*[37] evaluated 268 CD patients who had responded to infliximab induction. Of these patients, 197 patients (73.5%) had increased CRP levels at baseline. Ninety-two patients (46.7%) had CRP normalization (< 3 mg/L) at week 4, and another 29 (14.7%) had CRP normalization after 10 wk. Kaplan-Meier curves indicated that CRP normalization after 4 wk of therapy had long-term benefit ($P < 0.001$) out to 5 years with a PPV of 63%. Karmiris *et al*[38] reported similar findings for CD patients with baseline elevated CRP and normalization of CRP (< 3 mg/L) at both weeks 4 and 12 predicting less frequent discontinuation of adalimumab and longer sustained clinical benefit up to 2 years of follow up. Kiss *et al*[24] reported low CRP at week 12 (< 10 mg/L) as being a predictor of clinical remission at 52 wk (OR = 4.61, $P < 0.001$) during the first year of

Table 2 C-reactive protein values and correlating endoscopic scores

Ref.	Disease	Endoscopic tool	Endoscopic score (descriptor)	Calculation	CRP scores
Falvey *et al*[71]	CD	SES-CD	0-2 (inactive)	Mean (95%CI)	2.9 mg/L (1.8-4.6)
			3-6 (mild)		4.0 mg/L (2.6-6.1)
			7-15 (moderate)		5.1 mg/L (3.0-9.0)
			> 16 (severe)		22 mg/L (12.5-38.9)
Sipponen *et al*[88]	CD	CDEIS	< 3 (inactive)	Median (95%CI)	0.0 mg/L (0-21)
			3-9 (mild)		0.0 mg/L (0-26)
			9-12 (moderate)		8.5 mg/L (0-85)
			⩾ 12 (severe)		16.5 mg/L (0-211)
Schoepfer *et al*[77]	CD	SES-CD	0-3 (inactive)	Mean (range)	12 mg/L (3-94)
			4-10 (mild)		8 mg/L (3-53)
			11-19 (moderate)		23 mg/L (3-172)
			⩾ 20 (high)		40 mg/L (5-121)

CDEIS: Crohn's disease endoscopic index of severity; SES-CD: Simple endoscopic score for Crohn's disease.

adalimumab therapy.

Conversely, CRP levels are frequently elevated in patients who lose response to biologics[37]. Elevated CRP may be a sign of low drug level and a harbinger of ensuing loss of response and clinical relapse. In Jurgens *et al*[37], 57 CD patients who were responders to induction with infliximab had CRP and infliximab levels evaluated at week 14. In 75% of the patients who had clinical response after induction, a decrease in infliximab levels preceded loss of response by week 54. In 60% to 80% of patients with elevated CRP greater than 5 mg/L, the infliximab level was less than 1 μg/mL. CRP has also been shown to correlate better with low infliximab levels (< 1 μg/mL) than with clinical assessment using CDAI[39].

Higher CRP levels are also associated with an increased risk of surgery. In a Norwegian study, UC patients with a CRP above 23 mg/L at diagnosis were 4.8 times more likely to have surgery in the future (95%CI: 1.5-15.1, P = 0.02). At 1 year, UC patients with a CRP level greater than 10 mg/L were 3 times more likely to require surgery in the next 4 years (95%CI: 1.1-7.8, P = 0.02)[27]. CD patients with terminal ileitis were 6 times more likely to need future surgery if CRP levels at diagnosis were above 53 mg/L (95%CI: 1.1-31.9, P = 0.03).

PREDICTING POSTOPERATIVE RECURRENCE WITH CRP

Postoperative recurrence of CD is common. Up to 80% of CD patients will require surgery during their lifetime, and 70% of these patients will need a second surgery[40]. Predicting recurrence of CD after intestinal resection for strictures and fistulizing disease is difficult. Half of patients in clinical remission have ileocolonic ulcerations on endoscopic examination[41]. Treatment is tailored to the individual patient based on his or her risk of recurrence. The best biomarker for determining which postoperative CD patients are at highest risk of recurrence is not known. There are few studies dedicated solely to the evaluation of CRP and postoperative CD recurrence.

Previous studies report mixed results regarding the use of CRP for monitoring for postoperative recurrence in CD. Regueiro *et al*[42] reported a prospective cohort of 25 postoperative CD patients with CRP levels measured prior to surgery and then at 54 wk postoperatively. At 54 wk, there was no significant increase in CRP in patients who relapsed as compared to patients remaining in remission. CRP also did not correlate significantly with endoscopic scores in this study.

A smaller study has shown correlation between CRP and postoperative recurrence[43]. In 12 postoperative CD patients on infliximab without endoscopic or clinical recurrence after 3 years, infliximab was stopped; ten of 12 patients had endoscopic recurrence after 16 wk (Rutgeerts score > i2). After cessation of infliximab, CRP increased significantly in all patients compared to baseline (12.5 ± 4 mg/L *vs* 3.0 ± 1.4 mg/L, P < 0.001)[43]. Once infliximab was resumed in a dose-dependent fashion (1 to 3 mg/kg), the CRP significantly decreased (P < 0.0001). In this study, CRP significantly correlated with postoperative endoscopic recurrence, but again, the main limitation of this study is the small sample size. A recent study of 86 CD patients who underwent ileocolonic resection found a weak but significant difference in high sensitivity CRP (hsCRP) concentrations between patients in endoscopic remission and patients with recurrence (3.0 ± 0.7 mg/L *vs* 8.5 ± 1.4 mg/L; P = 0.0014)[44].

In summary, an elevated CRP has been shown to positively correlate with endoscopic disease activity and may predict ensuing relapse while a patient is in clinical remission. Therefore, a persistently elevated CRP in both CD and UC should prompt further investigation with further blood work, stool studies for infection, and endoscopic evaluation to evaluate for active disease. On the other hand, normal CRP levels in UC patients should be interpreted with caution as endoscopic disease may still be present. For predicting postoperative recurrence of CD, there is not strong data supporting the use of CRP or hsCRP.

Table 3 Endoscopic score correlation with fecal calprotectin

Ref.	Disease	Endoscopic tool	Correlation	*P* value
af Björkesten *et al*[25]	CD	SES-CD	$r = 0.560$	< 0.001
Sipponen *et al*[88]	CD	CDEIS	$r = 0.831$	< 0.001
Sipponen *et al*[49]	CD	SES-CD	$r = 0.642$	< 0.001
Sipponen *et al*[62]	CD	CDEIS	$r = 0.729$	< 0.001
Schoepfer *et al*[77]	CD	SES-CD	$r = 0.530$	< 0.010
Lobatón *et al*[74]	CD	CDEIS	$r = 0.722$[1]	< 0.001
			$r = 0.769$[2]	< 0.001
Lobatón *et al*[48]	UC	Mayo	$r = 0.741$[1]	< 0.001
			$r = 0.727$[2]	< 0.001
Takashima *et al*[67]	UC	Mayo	$r = 0.580$	< 0.0001
Røseth *et al*[66]	UC	Mayo	$r = 0.570$	< 0.0001
D'Haens *et al*[64]	UC	Mayo	$r = 0.623$	< 0.001
	CD	CDEIS	$r = 0.419$	< 0.001
	CD	SES-CD	$r = 0.490$	< 0.001

[1]FC-ELISA; [2]FC Q-POCT (quantitative-point-of-care test). CDEIS: Crohn's disease endoscopic index of severity; SES-CD: Simple endoscopic score for Crohn's disease; *r*: Spearman's rank correlation coefficient.

FC

First described in 1980, calprotectin is a 36 kilodalton inflammatory protein found in the cytosol of human neutrophils, macrophages, and monocytes[45,46]. Calprotectin comprises up to 60% of neutrophil cystolic proteins. The presence of calprotectin in the feces is directly proportional to neutrophil migration into the gastrointestinal tract during times of inflammation[12].

FC is a stable marker, resistant to degradation, that can be detected in stool for more than one week at room temperature[47]. Two FC assays are currently available: ELISA and a quantitative point-of-care-test (FC-QPOCT)[48]. Fecal lactoferrin, another stool neutrophil protein, is frequently paired with FC in clinical studies and generally has similar to slightly lower sensitivity and specificity when compared to FC[49-52].

Many gastrointestinal conditions can lead to elevations in FC concentrations including IBD, pouchitis, diverticulitis, malignancy, infections, nonsteroidal anti-inflammatory drug (NSAID) enteropathy, celiac disease, and microscopic colitis[53-55]. Though calprotectin is nonspecific and may be elevated in other gastrointestinal conditions, there is a substantial body of evidence supporting the use of FC in management of IBD.

Calprotectin levels have been reported to have low day-to-day variability in CD. Naismith *et al*[56] measured three consecutive days of FC levels in 98 patients with CD in clinical remission. An intraclass correlation (ICC) of 0.84 (95%CI: 0.79-0.89), low variability across patient samples, was reported. On the other hand, FC levels in UC patients have been shown to have high within-day variability[57]. Sampling the first bowel movement of the morning has been suggested to avoid falsely low measurements[58].

To further complicate matters, variations exist in FC levels depending on age. FC levels have been shown positively correlate with age in 320 normal adult subjects, ages 50 to 70[59]. Likewise, normal volunteers 60 years or older had higher FC levels than patients aged 10 to 59[60]. However, infants[61] and children less than 10 years old[60] have higher FC levels than adults.

FC CORRELATION WITH ENDOSCOPY

FC has been used to monitor patients during periods of quiescent disease. There is poor correlation between clinical assessment tools such as the CDAI with endoscopic inflammation in CD patients[3,49,62].

UC patients in clinical remission tend to have FC levels that positively correlate with endoscopic inflammation[63-66]. A study by Schoepfer *et al*[65] reported better correlation of endoscopic activity with FC than with other markers of inflammation including CRP, platelets, and serum leukocytes. In a recent study by Takashima *et al*[67], there was significant correlation of Mayo endoscopic scores with FC ($r = 0.58$; $P < 0.0001$) in 92 patients with UC.

In the meta-analysis by Mosli *et al*[28], FC predicted endoscopic activity with overall higher sensitivity than CRP, as expected. The pooled sensitivity and specificity of FC for endoscopically active IBD was 88% and 73%, respectively. When UC and CD were considered separately, UC exhibited equivalent sensitivity (88% *vs* 87%, respectively) but superior specificity (73% *vs* 67%) when compared to CD. An optimal FC cutoff of greater than 50 μg/g was calculated to signify endoscopically active disease. Stool lactoferrin had similar sensitivity and specificity (82% and 79%, respectively). A lactoferrin cutoff of greater than 7.25 μg/mL was calculated for endoscopically active disease.

In CD, clinical remission does not consistently correlate with FC levels[68,69]. Detecting subclinical inflammation is a high priority in CD to prevent long-term complications such as fibrostenotic strictures and perianal fistulae. However, endoscopic scores have been shown to correlate with FC levels in adults[25,49,62,64,70,71] and children[72,73] (Tables 3 and 4). In a group of 87 CD patients, D'haens *et al*[64] showed a significant correlation in adults between FC and CDEIS scores ($r = 0.419$, $P < 0.001$) and SES-CD ($r = 0.49$, $P < 0.001$) scores. Using receiver operating characteristic (ROC) curves, a cutoff of less than 250 μg/g correlated with endoscopic remission (CDEIS < 3) with high sensitivity (94.1%), moderate specificity (62.2%), and high negative predictive value (96.6%). Roseth *et al*[70] found that 44 out of 45 patients with a FC level < 50 mg/L had completely normal ileocolonoscopies. Moreover, by evaluating 18 of the stool samples from these same patients during previously active disease, the median FC level had been elevated to 3000 mg/L ($P < 0.0001$).

Isolated ileal CD impacts FC correlation with endoscopic scores. In a series of 87 consecutive ileocolonoscopies, there was a significant correlation with

Table 4 Fecal calprotectin values and correlating endoscopic scores

Ref.	Disease	Endoscopic tool	Endoscopic score (descriptor)	Calculation	Calprotectin scores
Falvey *et al*[71]	CD	SES-CD	0-2 (inactive)	mean (95%CI)	55 μg/g (25-123)
			3-6 (mild)		167 μg/g (97-288)
			7-15 (moderate)		366 μg/g (192-698)
			16+ (severe)		732 μg/g (338-1587)
Sipponen *et al*[49]	CD	SES-CD	≤ 3 (inactive-mild)	Median (range)	37 μg/g (13-166)
			> 3 (active)		686 μg/g (18-15326)
Sipponen *et al*[62]	CD	CDEIS	< 3 (inactive)	Median (range)	63 μg/g (11-869)
			3-9 (mild)		170 μg/g (17-2440)
			9-12 (moderate)		1014 μg/g (123-2284)
			> 12 (severe)		2066 μg/g (323-18575)
Schoepfer *et al*[77]	CD	SES-CD	0-3 (inactive)	mean (range)	104 μg/g (10-725)
			4-10 (mild)		231 μg/g (12-1009)
			11-19 (moderate)		395 μg/g (68-912)
			⩾ 20 (high)		718 μg/g (93-1327)
Lobatón *et al*[48]	CD	CDEIS	< 3 (endoscopic remission)	Median (range)	101.8 μg/g (30-1620.9)
			⩾ 3 (endoscopic activity)		1211.9 μg/g (122-1800)

CDEIS: Crohn's disease endoscopic index of severity; SES-CD: Simple endoscopic score for Crohn's disease.

FC and ileocolonic or colonic disease ($P < 0.001$)[49]. However, in isolated ileal CD, FC did not correlate with endoscopic SES-CD scores ($P = 0.161$) but did correlate with histology ($P < 0.001$). In a slightly larger study of 115 ileocolonoscopies, endoscopic findings exhibited excellent correlation with FC in ileocolonic disease ($r = 0.879$; $P < 0.001$) but only moderate correlation in ileal disease ($r = 0.437$; $P = 0.016$)[74]. Sipponen *et al*[75] found low sensitivity (59%) and moderate specificity (71%) when using FC to predict inflammatory small bowel lesions on subsequent capsule endoscopy.

In a more recent study of 44 patients with CD, 9 patients with isolated ileal disease had significantly lower FC levels when compared to patients with ileocolonic disease (297 ± 81 μg/g *vs* 1523 ± 97 μg/g, $P < 0.0001$)[76]. However, even though the levels of FC were significantly lower in isolated ileal disease, the FC levels were still elevated. Despite lower FC levels in patients with isolated ileal disease, there was still good overall correlation with SES-CD endoscopic scores ($r = 0.76$, $P < 0.0001$). Separate analysis of SES-CD correlation with FC levels in isolated ileal disease was not reported.

Schoepfer *et al*[77] described good correlation between FC levels and SES-CD for isolated ileal disease ($r = 0.649$, $P < 0.001$), but again, correlation between FC levels and SES-CD for ileocolonic disease was better ($r = 0.795$, $P < 0.001$).

In a study of children with CD, levels of FC were similar between isolated ileal disease and ileocolonic disease. In 60 newly diagnosed children with untreated CD, the median level of FC did not differ between children with isolated small bowel disease (47 patients) (2198 μg/g) and children with colonic involvement (2400 μg/g)[78].

PREDICTION OF RELAPSE USING FC

Despite continuous treatment, the majority of IBD patients will relapse. Evaluating which asymptomatic patients have smoldering subclinical inflammation is key to preventing further intestinal damage. Anticipating and altering treatment proactively helps prevent long-term complications. Approximately 35% of CD patients develop at least one fistula during the course of disease, and fistulas recur in one-third of patients[79]. Twenty-five percent of CD patients will have at least one small bowel stricture[80].

FC has been shown to correlate with histologic inflammation and to successfully predict relapses[81]. In a single-center, prospective study, 92 Crohn's patients in clinical remission (CDAI < 150) were observed for 12 mo. Ten patients (11%) relapsed by the end of one year. Median levels of FC were higher for relapsers than nonrelapsers (414 μg/g *vs* 96 μg/g, respectively; $P < 0.005$)[82]. In this study, Naismith *et al*[82] calculated that a FC greater than 240 μg/g was associated with a 12 times increased risk of relapse (Table 5). A meta-analysis of 6 studies with a total of 672 IBD patients (318 UC and 354 CD) reported a composite sensitivity of 78% (95%CI: 72-83%) and specificity of 73% (95%CI: 68%-77%) for predicting relapse using FC[83]. However, this meta-analysis did not report an optimal cutoff value for predicting relapse nor did the authors include CD patients with isolated ileal disease. Several studies have calculated optimal FC cutoffs to predict presence of endoscopic disease (Table 6).

Elevated FC levels have been reported to be present up to three months prior to clinical presentation of a UC flare[84,85]. De Vos *et al*[84] used FC levels to prospectively follow 87 patients with UC on maintenance infliximab therapy. FC levels were collected every 4 wk. Of these patients, 30 (34.4%) sustained deep remission (partial Mayo score < 3 and endoscopic Mayo score of 0 at one year) while 13 (14.9%) relapsed (Mayo score ⩾ 2 or need for change in treatment) during one year follow-up. Those patients in deep remission maintained very low FC levels (< 40

Table 5 Calprotectin cutoffs for predicting clinical relapse

Ref.	Disease	FC value	Relative risk	Sensitivity (%)	Specificity (%)
García-Sánchez *et al*[107]	UC	> 120 μg/g	6	80	60
	CD	> 200 μg/g	4		
Tibble *et al*[108]	UC/CD	> 50 μg/g	13	90	83
Kallel *et al*[109]	CD	> 340 μg/g	18	80	90.7
Naismith *et al*[82]	CD	⩾ 240 μg/g	12.18	80	74.4
Costa *et al*[110]	UC	> 150 μg/g	14	89	92
	CD	> 150 μg/g	2	87	43
D'Inca *et al*[103]	UC/CD	> 130 mg/kg	-	68	67

FC: Fecal calprotectin.

Table 6 Calculated fecal calprotectin cutoffs based on endoscopic score

Ref.	Disease	Endoscopic tool	Score	FC cutoff	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Guidi *et al*[111]	UC/CD	CDEIS	< 3 (mucosal healing)	121 μg/g[a]	79	57	-	-
D'Haens *et al*[64]	CD	CDEIS	< 3 (inactive)	250 μg/g	94.1	62.2	48.5	96.6
	UC	Mayo	1-3 (any inflammation)	250 μg/g	71	100	100	47.1
Sipponen *et al*[88]	CD	CDEIS	⩾ 3 (active)	200 μg/g	87	100	100	70
af Björkesten *et al*[25]	CD	SES-CD	0 (inactive)	94 μg/g	82	78	-	-
Lobatón *et al*[48]	UC	Mayo	0-1 (inactive-mild)[1]	250 μg/g[1,b]	73.5	89.7	86.2	79.5
			0-1 (inactive-mild)[2]	280 μg/g[2,b]	75.4	89.1	86	80.3
Takashima *et al*[67]	UC	Mayo	0 (inactive)	250 μg/g	82	62	61	83

[1]FC-ELISA; [2]FC-POCT; [a]$P = 0.038$; [b]$P < 0.001$ *vs* control. CDEIS: Crohn's disease endoscopic index of severity; SES-CD: Simple endoscopic score for Crohn's disease; FC: Fecal calprotectin.

mg/kg) with each sample analysis. Patients who flared exhibited elevated FC levels (> 300 mg/kg) beginning 3 mo prior to relapse. Interestingly, two consecutive FC levels greater than 300 mg/kg could predict relapse with a sensitivity of 61.5% and specificity of 100%.

Molander *et al*[85] monitored patients in endoscopic remission after infliximab cessation. Over one year of follow up after infliximab cessation, 15 UC patients (31%) and 34 CD patients (69%) relapsed. The patients who relapsed were found to have consistently elevated FC levels for a median of 94 d prior to relapse. There was a significant increase in FC levels at 2, 4, and 6 mo before endoscopic relapse ($P = 0.0014$, 0.0056, 0.0029, respectively). This suggests that the trend, rather than an isolated measurement, may be more valuable in predicting relapses.

Lasson *et al*[86] conducted a prospective, randomized, controlled study focused on altering therapy based on FC levels. They collected monthly FC levels in 91 UC patients with mild to moderate UC. If the FC value was higher than 300 μg/g on two consecutive measurements within one week, the dose of 5-aminosalicylates (5-ASAs) was escalated to try to prevent relapse. Of the patients with FC greater than 300 μg/g, the patients who had dose escalation of 5-ASAs had significantly reduced relapse rates as compared to patients in the control group ($P < 0.05$). In 18 of 28 patients (64.3%) in the dose escalation arm, their FC values dropped to less than 200 μg/g.

Calprotectin has been used to predict response to anti-TNF treatment during short-term follow-up periods. Several studies reported a significant correlation between decreases in FC and short-term endoscopic remission[50,87,88]. In one Dutch study of 53 patients with UC, patients in endoscopic remission at week 10 after infliximab induction had a steep decrease in week 2 FC levels as compared to pretreatment levels. At week 10, there was an excellent AUC for endoscopic remission and FC (AUC 0.91; 95%CI: 0.81-1.0)[87].

FC has also been used to predict long-term response to anti-TNFs. Molander *et al*[89] defined a cutoff of FC greater than 139 μg/g after completion of induction therapy to predict a risk of clinically active disease after 1 year for patients with IBD treated with either infliximab ($n = 42$) or adalimumab ($n = 18$). In pediatric IBD patients, long-term response (1.1 years median follow-up) after infliximab induction therapy was retrospectively linked to FC response between weeks 2 and 6[90]. Children who stopped therapy within the first year due to inadequate effect had higher median FC levels during induction than patients who responded (633 μg/g *vs* 219 μg/g, $P < 0.025$).

In children, the utility of FC varies greatly based on report. Sipponen *et al*[91] followed 72 children with IBD. The median age was 13. Twenty-five (35%) children clinically relapsed within the subsequent year with poor predictive value of FC for relapse (39.6% for FC > 100 μg/g; 42.9% for FC > 1000 μg/g). However, a systematic review of 34 pediatric studies determined that FC can be a marker of active inflammation with high sensitivity (range 94.4%-100%) and moderate specificity (71.9%-100%)[92]. As with adult studies, the

Table 7 Predicting postoperative recurrence with fecal calprotectin

Ref.	Type of recurrence	Follow-up time	FC value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Lasson *et al*[98]	Endoscopic	1 yr	100 μg/g	85	35	50	75
			200 μg/g	54	53	47	60
			250 μg/g	46	53	43	56
Wright *et al*[96]	Endoscopic	6 mo	135 μg/g	91	62	55	93
		18 mo	127 μg/g	88	67	58	91
Orlando *et al*[94]	Endoscopic	1 yr	200 mg/L	63	75		
Yamamoto *et al*[51]	Endoscopic	1 yr	140 μg/g	70	70	70	70
	Clinical		170 μg/g	83	93	83	93
Lobatón *et al*[74]	Endoscopic	Not specified	203 μg/g[1]	75	72	-	-
			283 μg/g[2]	67	72		
Boschetti *et al*[44]	Endoscopic	Within 18 mo	100 μg/g	95	54	93	77

[1]FC-ELISA test; [2]FC Q-POCT (quantitative-point-of-care test). FC: Fecal calprotectin.

cutoff range for detecting active IBD was large (50-275 μg/g).

PREDICTING POSTOPERATIVE RECURRENCE USING FC

Multiple studies have looked at FC for monitoring for postoperative recurrence of disease in CD with mixed results[44,52,74,93-97]. FC levels correlate with clinical indices such as the HBI[52] but not with the CDAI[51]. Several studies have reported that FC correlates with disease relapse both clinically[52] and endoscopically[44,51,74,96,97]. Papamichael *et al*[97] followed a group of 59 CD patients after ileocecal resection. Persistently elevated FC levels (> 60 μg/g) were found in 100% (15/59) of patients who had postoperative endoscopic recurrence (Rutgeerts score ⩾ i2) after ileocecal resection whereas CRP elevations (> 0.5mg/dL) were present in only half of the patients (P = 0.017).

Various cutoffs have been suggested to predict postoperative recurrence of disease (Table 7). Boschetti *et al*[44] reported a cutoff of 100 μg/g (sensitivity 95%, specificity 54%) to correlate with endoscopic recurrence (Rutgeerts score ⩾ i2) in 86 asymptomatic CD patients after ileocolonic resection. When evaluating correlation with Rutgeerts scores, FC performed better (r = 0.65, P < 0.001) than hsCRP (r = 0.34, P = 0.0016). This study excluded patients with perianal disease. Stool samples were collected one week prior to endoscopic evaluation.

Yamamoto *et al*[93] collected stool samples from 20 asymptomatic postoperative CD patients at the beginning of the study then followed them for 1 year. The mean duration from surgery to endoscopic evaluation was 7.2 mo. A calculated FC cutoff of 140 μg/g predicted endoscopic recurrence whereas a cutoff of 170 μg/g predicted future clinical recurrence.

On the other hand, several studies reported that calprotectin was not consistent in predicting recurrence after surgery. Scarpa *et al*[95] retrospectively studied 63 CD patient FC levels for a median of 40.5 mo after surgery. There was no significant difference in FC levels between patients who remained in clinical or endoscopic remission and patients who had a recurrence of disease. The authors cited the limited correlation of the CDAI with inflammation and the lag in time between stool sample collection and endoscopy as possible explanations for lack of significance. However, there was a significant difference in FC levels between patients who required further ileocolonic resection and patients who did not need more surgery (P = 0.04), but this result is limited by small sample size (5 patients required further surgery). Lasson *et al*[98] reported a nonsignificant trend towards lower FC levels in patients in remission and higher FC levels in patients with endoscopic recurrence at one year postoperatively (P = 0.25). The small sample size of 30 patients and follow-up time were limitations to the study; one patient from the remission group ended up having a flare 6 mo after the study ended.

In a more recent prospective, randomized control trial in Australia and New Zealand, CD patients who underwent intestinal resection were followed up to 18 mo postoperatively. The median FC level decreased from 1347 μg/g prior to surgery to 166 μg/g at 6 mo postoperatively. Patients with endoscopic disease recurrence had higher median FC levels than patients who maintained remission (275 μg/g *vs* 72 μg/g, respectively; P < 0.001)[96]. Of note, CRP levels and clinical CDAI scores did not correlate with CD recurrence or severity of disease. A cutoff FC level of greater than 100 μg/g indicated endoscopic recurrence with a sensitivity of 89%, specificity of 58%, and negative predictive value of 91%. The high NPV of 91% suggests that endoscopy may be able to be avoided or deferred in patients with FC measurements less than 100 μg/g.

Overall, FC is a useful biomarker that is more specific for intestinal inflammation than CRP. FC correlates better with ileocolonic disease than with isolated ileal disease. FC is useful in predicting clinical and endoscopic relapse while in clinical remission, as well as monitoring response to medical therapy. Evidence suggests that monitoring for postoperative

recurrence is more reliable with FC than CRP.

NEW APPLICATIONS: FECAL IMMUNOCHEMICAL TEST

Fecal immunochemical test (FIT) is an alternative modality being considered for use in IBD, much less utilized than FC or CRP. Quantitative FIT testing measures stool hemoglobin concentrations using an antibody specific for human hemoglobin[99]. FIT has mainly been publicized as a method for screening for colonic neoplasia[100]. As shown in a capsule endoscopy study, positive FIT tests can be explained by isolated small bowel lesions without colonic pathology[101].

Specifically relating to IBD, FIT has been used to predict mucosal healing in patients with UC with a 92% sensitivity and 71% specificity[99]. In a recent prospective trial from Japan, FIT was compared with FC to evaluate for mucosal healing in 92 patients with UC[67]. Of the 105 colonoscopies done, 77 (73%) were in patients in clinical remission. However, only 42% of colonoscopies demonstrated complete mucosal healing (Mayo score 0). Both the FIT and FC levels significantly correlated with the Mayo score. There was also significant correlation between the FIT values and FC levels (Spearman's correlation coefficient 0.64, $P < 0.0001$). The sensitivity and specificity of FIT for predicting mucosal healing was 95% and 62%, respectively, for a fecal hemoglobin concentration less than 100 ng/mL. Comparatively, for a FC cutoff less than 250 μg/g, there was lower sensitivity at 82% and equivalent specificity at 62% for predicting mucosal healing.

FIT is currently less expensive than FC. There may be a future role for FIT in disease monitoring in IBD, but more trials are needed.

CONCLUSION

Our goals of treating IBD patients have evolved over the past few years to include mucosal healing in addition to clinical remission. Ideally, by monitoring disease activity via noninvasive blood or stool markers, we may be able to identify patients with subclinical disease activity and thereby optimize treatment prior to a clinical flare.

Furthermore, the practice of medicine is changing in the face of healthcare spending reforms. Cost cannot be overlooked. In the future, procedures such as colonoscopy may not always be cost-effective or time-efficient. Consistently reliable, noninvasive assays to evaluate subclinical disease activity will be valuable for determining which endoscopic evaluations may be deferred.

CRP and FC have emerged as two of the most commonly used biomarkers to evaluate for subclinical disease activity in IBD. There are pros and cons to keep in mind when ordering each biomarker.

CRP is low-cost, easy to obtain with simple bloodwork, and quick to deliver data. CRP has been reported to have modest correlation with endoscopic and clinical findings, generally better with CD than UC. The major downsides to CRP are its lack of specificity for intestinal inflammation and moderate false negative rate. Genetic variations in CRP likely contribute to its overall lower sensitivity[23].

CRP does not reliably predict postoperative recurrence in CD. Just as postulated in active UC with normal CRP, early inflammation in postoperative recurrence may not be detectable using CRP due to lack of transmural inflammation. Existing data suggests that FC is a more sensitive measure of recurrent intestinal inflammation in postoperative CD patients.

FC is more expensive but is a more specific marker of intestinal inflammation. FC tends to correlate better with endoscopic findings in IBD than CRP, except in cases of isolated small bowel CD where FC levels are lower. CRP still plays a role in evaluation of isolated small bowel disease.

When considering the utility of FC in predicting endoscopic relapse in IBD and postoperative recurrence in CD, a noteworthy limiting factor for real-world use is the wide variation in defined cutoffs for inactive *vs* active disease (Tables 4-6). Generally, very high levels of FC indicate active disease, and FC levels less than 50 μg/g indicate inactive disease. However, many clinicians may find themselves questioning the significance of moderately elevated or upper limit of normal FC values.

The type of assay used (ELISA *vs* FC-QPOCT) may contribute to the wide range of cutoffs reported. Moreover, variations in calprotectin extraction methods can result in different FC quantitations from the same stool sample. During a quality assurance study, Whitehead *et al*[102] reported an average of 7.8% to 28.1% under-recovery of FC with different ELISA assays.

Heterogeneity in study design also may be a factor affecting FC cutoff levels. The definition of endoscopically inactive disease varies among studies. Also, time points for stool collection vary widely among studies. For example, one study may collect a stool sample on the day prior to colonoscopy[74] whereas another study may collect stool at an unspecified time point prior to clinical flare[103]. In the postoperative studies, there is variation in clinical status (remission *vs* symptomatic), disease phenotypes included, timing of postoperative endoscopic evaluation, as well as length of study follow up.

Perhaps a "one size fits all" approach does not pertain to calprotectin cutoffs in IBD. Optimal cutoffs may differ by disease (UC, CD), distribution of inflammation, age of patient, brand of assay used. For example, given that FC levels have been shown to be lower in isolated ileal disease, lower cutoff values may be needed for ileal CD without colitis. Also, in adults, the increase in FC with age may also need to be taken into account. Future investigations are needed to

further define these cutoffs.

In our practice, we use both CRP and FC to monitor patients in clinical remission. FC is preferred but not always sent due to cost and lack of coverage by certain insurance carriers. If FC is less than 50 μg/g, we do not routinely further evaluate the patient, whereas if the FC is greater than 250 μg/g, we rule out infection with stool studies and then consider an endoscopic evaluation. If the FC level is between 50 and 250 μg/g, we like to complete a colonoscopy at that time to correlate levels with endoscopic appearance. Still, in most cases, since levels vary from person to person, we find it most helpful to make treatment decisions based on a combination of FC, CRP, and endoscopic findings.

In the postoperative setting, we do not use CRP because of the lack of efficacy. We send FC levels at month 3. If elevated, we evaluate with colonoscopy. If normal, a colonoscopy is performed between 6 and 12 mo after resection.

Due to the nature of clinical research, most clinical studies focus on short-term patient responses to treatments. Less is known about long-term results of chronic biologic and immunomodulators therapies. The ultimate goal of therapy in IBD patients is to minimize the long-term sequelae of chronic inflammation while avoiding exposing the patient to unnecessary risks such as infection and neoplasia[104]. In 2009, the STORI trial evaluated stopping infliximab in patients on combination therapy who had been in steroid-free clinical remission for at least 6 mo[105]. Other studies have evaluated stopping immunomodulators while patients are maintained solely on infliximab[106]. The optimal duration of these drug holidays is unknown. With future trials underway evaluating the safety and logistics of withdrawing therapy, the role of monitoring clinically silent disease will be key in differentiating those patients who will remain quiescent and those who should re-escalate therapy.

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TOPIC HIGHLIGHT

2015 Advances in Inflammatory Bowel Disease

From the surface to the single cell: Novel endoscopic approaches in inflammatory bowel disease

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Abstract

Inflammatory bowel diseases (IBD) comprise the two major entities Crohn's disease and ulcerative colitis and endoscopic imaging of the gastrointestinal tract has always been an integral and central part in the management of IBD patients. Within the recent years, mucosal healing emerged as a key treatment goal in IBD that substantially decides about the clinical outcome of IBD patients, thereby demanding for a precise, timely and detailed endoscopic assessment of the mucosal inflammation associated with IBD. Further, molecular imaging has tremendously expanded the clinical utility and applications of modern endoscopy, now encompassing not only diagnosis, surveillance, and treatment but also the prediction of individual therapy response. Within this review we describe novel endoscopic approaches and advanced endoscopic imaging methods for the diagnosis, treatment and surveillance of IBD patients. We begin by providing an overview over novel and advanced imaging techniques such as magnification endoscopy and dye-based and dye-less chromoendoscopy, endomicroscopy and endocytoscopy. We then describe how these techniques can be utilized for the precise and ultrastructural assessment of mucosal inflammation and dysplasia development associated with IBD and outline how they have enabled the endoscopist to gain insight onto the cellular level in real-time. Finally, we provide an outlook on how molecular imaging has rapidly evolved in the recent past and can be used to make individual predictions about the therapeutic response towards biological treatment.

Key words: Gastrointestinal endoscopy; Crohn's disease; Ulcerative colitis; Inflammatory bowel diseases; Colon; Colorectal neoplasms

Core tip: Within this review we describe novel endoscopic techniques for the diagnosis, treatment and surveillance of inflammatory bowel diseases (IBD)

patients. We begin by providing an overview over advanced imaging techniques such as magnification endoscopy, dye-based and dye-less chromoendoscopy, endomicroscopy and endocytoscopy. We then portray how these techniques provide insights on cellular level in real-time and how they can be utilized for the precise and ultrastructural assessment of mucosal inflammation and dysplasia development in IBD. Finally, we review how molecular imaging has rapidly evolved in the recent past and can now be used to make individual predictions about the therapeutic response towards biological treatment.

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INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) belong to the family of idiopathic inflammatory bowel diseases (IBD) in which an excessive mucosal immune response towards the complex enteric microbiota in a genetically predisposed host is believed to play a key role in disease pathophysiology[1-4]. It is well accepted that the chronic inflammatory stimulus within the gastrointestinal tract is associated with an increased risk for developing colitis associated cancer (CAC) in both, UC and CD[5] and the individual risk for colon cancer increases with the duration, severity and anatomic extent of colitis[6-10]. The close association between disease duration and the development of CAC represents the rationale for recommending regular surveillance endoscopy starting 6 to 8 years after first manifestation of the disease in current European and United States guidelines[11,12]. Despite the lack of randomized controlled trials directly assessing the reduction of CAC by surveillance colonoscopy, a large number of case series[13-16] and case-control studies[17-19] provided evidence of the clinical benefit of surveillance colonoscopy for IBD patients. However, dysplasia and intraepithelial neoplasia are frequently missed during routine white-light endoscopic examinations[20] and at the same time, random biopsies have a low yield for dysplasia detection[20,21].

The discovery that dye-based chromoendoscopy (*e.g.*, with methylene blue) with targeted mucosal biopsies is superior for dysplasia detection in IBD patients[20,21] has led to the rapid evolvement of advanced endoscopic imaging techniques such as digital (*i.e.,* FICE, i-scan, SPIES) or optical [*i.e.,* narrow band imaging (NBI), Compound band imaging (CBI)] dye-less chromoendoscopy which offer the advantage of enhancing mucosal vascular and mucosal surface pattern morphology by just pushing a button on the handle of the endoscope thereby reducing time and costs associated with conventional dye-based chromoendoscopy[22,23] (Table 1).

Apart from the detection of early colorectal cancer, endoscopic assessment of degree and severity of mucosal inflammation is another equally important aspect in the management of IBD patients. In this regard, mucosal healing has emerged as a key treatment goal in IBD in the recent past that predicts sustained clinical remission and resection-free survival of patients[24]. Hence, the precise assessment of intestinal inflammation is of pivotal importance for the management of IBD patients and advanced endoscopic imaging techniques including dye-less chromoendoscopy, endocytoscopy and confocal laser endomicroscopy have been shown to allow precise and ultrastructural characterization of the inflammation within the gut. Finally, by combining endoscopic imaging with the visualization of single molecular targets crucially involved in disease pathogenesis, *in vivo* endoscopic prediction of therapeutic response before the actual commencement of therapy is no longer a keen wish for distant future, but close to be ready for being integrated into daily practice. In this review we describe how novel and advanced endoscopic imaging techniques have been utilized for the diagnosis and surveillance of CAC and mucosal inflammation in IBD patients and follow a semantic structure "From the surface to the single cell". Thus, we begin by reviewing imaging techniques that visualize the intestinal surface such as chromoendoscopy and subsequently discuss endoscopic approaches that go deeper within the intestinal layer and are capable of visualizing the submucosal architecture and single cells such as endocytoscopy and confocal endomicroscopy. Finally, we provide an outlook on how labelling molecular pathways and targets combined with endoscopy can be utilized to make predictions about therapeutic responses, thereby tremendously expanding the repertoire of modern endoscopy.

TECHNIQUES BEHIND ADVANCED ENDOSCOPIC IMAGING

Magnification endoscopy and chromoendoscopy

Magnification endoscopy utilizes a movable lens to vary the degree of magnification thereby allowing to magnify the mucosa of the gastrointestinal tract from 6-fold up to 150-fold[25]. In one of the earliest studies, magnification endoscopy has been shown to be able to differentiate true neoplasms from non-neoplastic colonic lesions, thereby providing an accurate instantaneous prediction of the histology of colorectal tumorous lesions[25]. This observation in colorectal polyps has now been dramatically extended to other neoplastic and non-neoplastic diseases in the upper and lower gastrointestinal tract, and especially

Table 1 Techniques and modes of advanced endoscopic imaging with advantages and indications

Endoscopic technique	Modes	Advantages	Disadvantages	Indications
White light endoscopy (WLE)	Standard definition colonoscopy (SD)	Widely spread	No sufficient discrimination between inflammation and dysplasia	Routine assessment of mucosal inflammation
	High definition colonoscopy (HD)	Can detect significantly more dysplastic lesion in IBD than SD[108]	Increased costs compared to SD	in combination with DBC: cancer surveillance of IBD patient[109,110]
Dye based chromo-endoscopy (DBC)	Indigo-carmine (0.8%)[109,110]	Superior for the detection of dysplastic lesions in IBD[20,21,55,56,109,110]	Increase in time and effort, dye-pooling	Method of choice for cancer surveillance in IBD[12,34,35, 109,110]
	Methylene blue (1%)[109,110]			
Dye less chromo-endoscopy (DLC)				
Optical DLC	NBI CBI	Readily available (push-of-a-button technologies)[22,31,66] improved prediction of disease extent and disease activity compared to WLE[67-69,71]	NBI: results for dysplasia detection in IBD heterogenous	NBI: not recommended as a replacement for DBC for cancer surveillance in IBD[109,110]
Digital DLC	i-scan FICE SPIES		i-scan: no data for dysplasia detection in IBD yet	
Confocal laser endomicroscopy (CLE)	pCLE iCLE	Real time histologic imaging with 1000-fold magnification, potentially improved delineation of degree and extent of mucosal inflammation[82]	Time- and cost-intensive procedure, expert skills required[22,31]	No routine indications
Endocytoscopy (EC)		Real time histologic imaging with up to 1390-fold magnification, potentially improved delineation of degree and extent of mucosal inflammation, can distinguish single inflammatory cells[88]	Time- and cost-intensive procedure, expert skills required[22,31,111]	No routine indications

CBI: Compound band imaging; DBC: Dye-based chromoendoscopy; DLC: Dye-less chromoendoscopy; FICE: Fuji intelligent color enhancement; HD: High definition; IBD: Inflammatory bowel diseases; iCLE: Integrated confocal laser endomicroscop; NBI: Narrow band imaging; SD: Standard definition; SPIES: Storz professional image enhancement systems; pCLE: Probe based confocal endomicroscopy.

in combination with chromoendoscopy, magnification endoscopy can be utilized for the precise diagnosis of a variety of diseases including dysplasia and early cancer in the esophagus, stomach and colorectum as well as intraepithelial neoplasia and disease extent in UC[26-32].

Chromoendoscopy encompasses dye-based chromo-endoscopy (DBC) and dye-less chromoendoscopy (DLC) and enhances the mucosal architecture and/or submucosal microvasculature by the use of various dyes (DBC) or endoscopic optical and computer-based color programs (DLC). This contrast enhancement of the mucosal layer often results in the improved detection of lesions that are otherwise subtle or even invisible in conventional white-light endoscopy.

DBC uses different dye agents which are divided into absorptive agents (Lugol, methylene blue, toluidine blue, and cresyl violet), contrast agents (indigo carmine, acetic acid) and reactive staining agents (congo red, phenol red), all of which are mostly applied *via* standard spraying or plain biliary ERCP catheters[33]. As outlined below, DBC has been shown to improve detection of dysplasia in IBD, and chromoendoscopy is recommended as the preferred modality for surveillance in patients with colonic IBD by the British Society of Gastroenterology[34] and the European Crohn's and Colitis organization[35]. However, DBC also requires increased effort, skill, time, and costs. These confinements associated with the use of traditional dye agents have finally led to the development of DLC techniques.

DLC is further subdivided into optical chromo-endoscopy [Narrow band imaging (NBI), Olympus, Japan; Compound band imaging (CBI), Aohua, Shanghai, China] and digital chromoendoscopy [i-scan, Pentax, Tokyo, Japan; Fujinon Intelligent Color Enhancement (FICE), Fujifilm, Tokyo, Japan; Storz Professional Image Enhancement Systems (SPIES), Karl Storz, Tuttlingen, Germany]. Optical DLC such as NBI utilizes optical filters within the light source of the endoscope to narrow the bandwidth of spectral transmittance, thereby enhancing and facilitating the visualization of blood vessels. Digital DLC such as i-scan and FICE uses a digital postprocessing algorithm that reconstructs the endoscopic image from the video processor in real time resulting in an improved contrast of the capillary patterns and enhancement of the mucosal surface pattern morphology[22,33]. Representative images for the enhanced visualization and delineation of the mucosal surface pattern by dye-based chromoendoscopy, and the mucosal surface and vascular pattern by NBI and

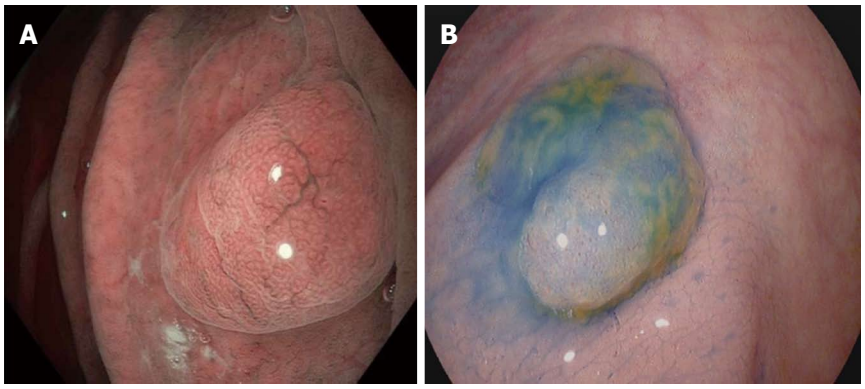

Figure 1 Dye-based chromoendoscopy and optical chromoendoscopy in the gastrointestinal tract. Left picture: Optical dye-less chromoendoscopy with narrow band imaging (NBI) is based on the utilization of optical filters within the light source of the endoscope to narrow the bandwidth of spectral transmittance, thereby enhancing and facilitating the visualization of blood vessels. As exemplified on a fundic gland polyp in the stomach, NBI allows a clear delineation of the mucosal surface pit pattern architecture. Right picture: Dye-based chromoendoscopy with indigo carmine. Indigo carmine is a blue contrast agent that is used primarily in the colon for enhancing the detection or differentiation of colorectal neoplasms. As shown for a small colon polyp here, application of indigo carmine via a spraying catheter enhances the contrast and allows to visualize the pit pattern and to delineate mucosal irregularities.

i-scan are shown in Figures 1 and 2, respectively. Importantly, both optical and digital DLC are simple "push-of-a-button" techniques that are readily available during the endoscopic examination. Thus, compared to dye-based chromoendoscopy, DLC offers the great advantage of dye-enhanced mucosal imaging without the efforts in time and costs of applying contrast agents during ongoing endoscopy. Further, data derived from the *in vivo* assessment of colorectal polyp histology impressively demonstrated that DLC can be readily learned even by "non-expert" endoscopists[36-38]. Hence, endoscopists with varying levels of experience can accurately use digital chromoendoscopy after a single training session[39,40] with comparable diagnostic accuracies between non-expert and expert endoscopists[41].

Confocal laser endomicroscopy

Confocal laser endomicroscopy is a technique allowing to obtain images at the (sub)cellular level[42], and since its introduction in 2003 confocal laser endomicroscopy (CLE) has rapidly emerged as a powerful technique that enables precise histologic real time *in vivo* imaging of various diseases[43-47]. Technically, CLE is based on the emission of a low power blue laser into the tissue after topical (acriflavine hydrochloride, cresyl violet) or systemic (fluorescein sodium) administration of contrast agents. The emitted light is then reflected from the tissue and refocused on the detection system by the same lens, leading to microscopic imaging at 1000-fold magnification in real time. As shown in healthy mucosa in Figure 3, CLE allows a clear visualization of the colonic crypt architecture, single cells within the lamina propria and the microvasculature within the colon[45]. Currently, two FDA-approved and CE-certified CLE devices are available and used in clinical routine[48]: (1) a probe based CLE system that can be inserted into the accessory channel of any standard endoscope (pCLE, Cellvizio, Mauna Kea Technologies, Paris, France); and (2) an integrated system in which the CLE probe is integrated into the distal end of a high-resolution endoscope ("integrated", iCLE; Pentax, Tokyo, Japan). Both system use a blue laser light source that delivers an excitation wavelength of 488 nm, and light emission from the tissue is detected at wavelengths between 205 and 585 nm. The iCLE-system collects images at a manually adjustable scan rate of 1.6 frames per second with a resolution of 1024 × 512 pixels, or at 0.8 frames per second with a resolution of 1024 × 1024 pixels. The depth of scanning can be dynamically adjusted ranging from 0 to 250 μm and the laser power can be manually adjusted between 0 and 1000 μW. The optical slice thickness is 7 μm, with lateral and axial resolution of 0.7 μm and a confocal image field of view of 475 μm × 475 μm. Since the laser probe is integrated into the endoscope, the accessory channel of the endoscope can still be used.

The pCLE system is based on stand-alone confocal probes, and specific probes available for different indications throughout the entire gastrointestinal tract are available. Probe-based CLE utilizes a fixed laser power and a fixed imaging plane depth for image acquisition. Lateral resolution ranges between 3.5 μm and 1 μm, resulting in a field of view of 600 μm-240 μm, depending on the confocal probe used. Images are acquired at 12 frames/s, leading to real-time videos of the intestinal mucosa and single video frames either in real time or post processed with an increased field of view (4 mm x 2 mm) can be reconstructed using a special computer algorithm (Mosaicing, Mauna Kea Technologies, Paris, France). Probe based CLE in IBD is mostly being performed by using the ColoFlex UHD probe which requires a 2.8 mm working channel. Hence, these probes can be fitted through the working channel of most endoscopes used in clinical practice.

Depending on the clinical question and the scenario in which they are used, both CLE-systems offer unique

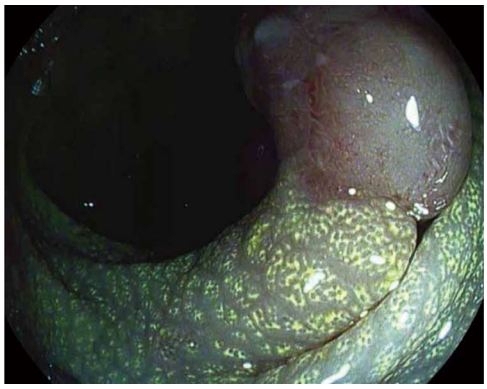

Figure 2 Digital dye-less chromoendocopy with i-scan in the lower gastrointestinal tract. i-scan uses a digital postprocessing algorithm that reconstructs the endoscopic image from the video processor in real time resulting in improved contrast of the capillary patterns and enhancement of the mucosal surface pattern morphology as exemplified on a adenomatous polyp in the colon. As a result of an accumulation of lipid-filled macrophages within the lamina propria, the mucosa adjacent to the polyp exhibits a chicken skin mucosa on digital chromoendoscopy.

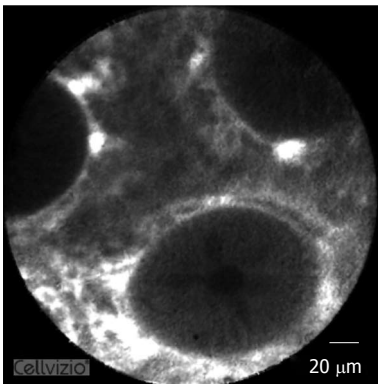

Figure 3 Confocal laser endomicroscopy in the lower gastrointestinal tract. Confocal laser endomicroscopy (CLE) is based on the emission of a low power blue laser into the tissue after topical or systemic administration of contrast agents. The emitted light is then reflected from the tissue and refocused on the detection system by the same lens, leading to microscopic imaging at 1000-fold magnification in real time. As shown in healthy colonic mucosa in this picture, CLE allows a clear visualization of the colonic crypt architecture, single cells within the lamina propria and the microvasculature within the colon.

advantages and specifications. Advantages of the integrated system are its higher resolution and the possibility to alter the laser power and imaging plane depth, whereas the pCLE system allows *ad hoc* usage in existing endoscopes and enables real time video recording.

Endocytoscopy

Endocytoscopy (EC) allows *in vivo* microscopic imaging of the GI tract with an magnification ranging from 340-fold up to 1390-fold[49-51] and is based on the principle of contact light microscopy. EC utilizes a fixed-focus, high-power objective lens that projects highly magnified images from a sampling site onto a charge-coupled device[49-51]. The depth of field ranges from 0 to 50 μm and therefore only allows visualization of the very superficial mucosal layer. EC requires thorough mucolysis which can be performed with N-acetyl-cysteine. Further, prestaining of the mucosa with an absorptive agent such as methylene blue, toluidine blue, or cresyl violet is required. Optimal endocytoscopic mucosal imaging can be obtained with 1% methylene blue in the oesophagus and with 0.25% toluidine blue in the stomach and colon after 60 s of exposure to the dye[52].

In fact, a combination of different dye agents is often used to generate optimal tissue contrast and imaging modalities[53]. With the use of absorptive agents *via* spraying catheters, repeated staining of the mucosa may be needed when the clinical scenario requires extended visualization[50]. Endocytoscopy images are displayed on a monitor at 30 frames per second, which corresponds to the frame rate during routine high-resolution video endoscopy.

Currently, two systems of endocytoscopes are available[50]. Similar to the CLE devices, endocytoscopy devices can be integrated into the distal tip of a standard endoscope (iEC) or utilized as a probe that is advanced through the working channel of a standard endoscope (pEC). Endoscope-based instruments use two different lenses and integrate the EC component within upper (103 cm in length) and lower (133 cm in length) endoscopes and provide a 580x-fold image magnification on a 19-inch monitor, in addition to having conventional optical magnification and narrow band imaging capabilities. Recently, another endocytoscopy system (GIF-Y0002) was introduced consisting of only one lens that allows continuous increase of zooming power from the conventional endoscopy level up to 380-fold (tissue field of view, 700 mm x 600 mm) using a hand lever. Using digital magnification (x 1.6), the magnifying power can be increased to 600-fold, providing a tissue field of view measuring 440 mm x 380 mm[51,54]. For the first time, this new endoscope-generation enables continues magnification from standard overview to endocytoscopy therefore representing an "all-in-one" scope.

Currently, two different probe-based EC devices exist, providing either 450-fold (XEC 300F) or 1390-fold (XEC 120 U) magnification images on a 19-inch monitor[50,51]. The probes are 380 cm in length and 3.2 mm in diameter thus requiring an accessory channel of 3.7 mm. The horizontal observation field is given with 300 μm x 300 μm (0.09 mm^2) for the 450-fold magnification probe and with 120 μm x 120 μm for the 1390-fold magnification probe. Similar to what is already discussed with different CLE devices, one of the major advantages of the probe-based EC system lies in its *ad hoc* usability in already existing endoscopes whereas the integrated EC devices allow to simultaneously take biopsies and thus to directly compare EC imaging with histopathological results.

ROLE AND APPLICTIONS OF ADVANCED ENDOSCOPIC IMAGING IN IBD

Surface level: Dye-based and dye-less chromoendoscopy

In one of the earliest prospective randomized trials on the relevance of dye-based chromoendoscopy (DBC) for the assessment of mucosal inflammation and dysplasia in UC, Kiesslich *et al*[21] directly compared DBC and conventional colonoscopy in a large cohort of UC patients. Importantly, DBC with methylene blue not only permitted a more accurate diagnosis of the extent and severity of the inflammatory activity in UC compared with conventional colonoscopy, but also significantly improved the early detection of intraepithelial neoplasia and CAC. Another "back-to-back" study evaluated pancolonic indigo carmine staining (0.1%) for the detection of UC-associated dysplasia[20]. As shown in this study, DBC with indigo carmine led to a higher dysplasia detection rate while at the same time reducing the total amount of biopsies[20]. Consistent with these results, another prospective trial also included patients with Crohn's colitis (CC), and similarly, in both UC and CC, targeted biopsies with dye spray (methylene blue) detected significantly more dysplasia than random biopsies that were taken without the utilization of dye[55]. As shown in a recent meta-analysis of six randomized controlled trials, dye-based chromoendoscopy has a medium to high sensitivity and a high diagnostic accuracy for dysplastic lesions in UC[56] and the typical features of UC associated dysplasia on DBC (and conventional endoscopy) have been summarized by Matsumoto *et al*[57] from the Hyogo College of Medicine in Japan. Since white-light endoscopy exhibits only low interobserver agreement in differentiating dysplastic from non-dysplastic lesions during colitis surveillance, current guidelines recommend chromoendoscopy with targeted biopsies as the surveillance procedure of choice for appropriately trained endoscopists, whereas white-light endoscopy with random biopsies (quadrant biopsies every 10 cm) remains a reasonable alternative for cancer surveillance in IBD patients[11,12,58].

Since DBC is also associated with a potential increase in examination time, costs and overall effort, a recent study evaluated whether DBC is cost-effective for colorectal cancer surveillance in UC patients. Interestingly, DBC with targeted biopsies is not only more effective but also less costly compared to conventional white-light endoscopy with random biopsies[59]. In its totality, this profound evidence on the superiority of DBC for the detection of colitis-associated neoplasia, together with the knowledge of a cumulative CRC risk in UC patients of 18% after 30 years of disease[7], have led to the recommendation to perform chromoendoscopy with targeted biopsies as the surveillance procedure of choice in IBD patients in US and European guidelines[11,12,34,35].

The first case in which optical DLC was used to help in identifying colitis associated neoplasia was a 63 year old man with longstanding ulcerative colitis and a previous history of dysplasia associated lesions or masses (DALM). In this patient it was shown for the first time that visualization of the pit pattern and the vascular pattern intensity by NBI might help in DALM detection and to distinguish dysplastic from non-dysplastic mucosa in ulcerative colitis[60]. Especially the capillary vasculature in dysplastic lesions exhibited a higher vascular pattern and appeared darker on NBI compared to adjacent normal mucosa[60]. Since then, various trials have studied the potential of NBI to assess mucosal inflammation and colitis associated preneoplastic and neoplastic changes, with so far mixed results. In one of the earliest reports, the Amsterdam group compared the accuracy of NBI with standard colonoscopy for the detection of neoplasia in patients with longstanding ulcerative colitis[61]. Although more suspicious lesions were found during DLC with NBI, the sensitivity of NBI for neoplasia detection was similar to conventional white-light endoscopy[61]. Soon thereafter, the same group assessed the value of NBI for surveillance in UC in two other studies[62,63]. In these studies, pit pattern analysis of neoplastic lesions exhibited only a moderate accuracy for the prediction of histology[62] and also NBI did not improve the detection of UC associated neoplasia compared to high-definition endoscopy[63]. Nevertheless, NBI has been shown to be equally effective in detecting UC associated intraepithelial neoplasia compared to conventional dye-based endoscopy and exhibited a reduced false-positive biopsy rate and a similar true-positive rate[64]. However, the high miss rate with NBI, as pointed out by the authors themselves, makes NBI not advisable as the standard technique to detect dysplasia in patients with long-standing IBD[64] and clearly, higher powered studies are needed to address this question[65,66].

The role of dye-less chromoendoscopy to assess mucosal inflammation associated with IBD has also been studied. In one of the earliest reports, Kudo *et al*[67] analyzed the mucosal vascular pattern (MVP) in patients with asymptomatic or mildly active UC using NBI and HD white-light endoscopy. The authors found that areas with obscure MVP on NBI exhibit increased numbers of acute inflammatory cell infiltrates, goblet cell depletion and basal plasmacytosis and that evaluation of the MVP with NBI yielded a more precise determination of acute microscopic inflammation in patients with quiescent UC[67]. The typical appearance of active UC and inactive, quiescent disease on NBI have been summarized by the same group of authors[68]. In addition to that, another pilot study on 14 IBD patients was able to demonstrate that areas that appear normal on WLE, but positive on NBI (as defined by a stronger capillary vascular pattern), exhibit an increased leukocyte infiltrate

and a significantly increased microvessel density on immunohistology, thus providing first evidence that NBI might allow *in vivo* imaging of intestinal neoangiogenesis in IBD patients[69].

Data on the relevance of digital DLC for the assessment of mucosal inflammation in IBD patients are limited. To date, only one study evaluated FICE in IBD patients and showed that FICE is not helpful to improve the detection or delineation of ulcers and erosions in CD[70]. Just recently, a study on 78 IBD patients that were randomized to receive either HD white-light endoscopy or HD endoscopy with i-scan, was able to demonstrate that i-scan allows a considerably improved prediction of disease extent and disease activity compared to white-light endoscopy (i-scan: 92% and 90% *vs* WLE: 49% and 54%)[71]. Of note, examination time was not different between WLE and i-scan, consistent with the idea that dye-less chromoendoscopy is a push-of-a-button technology that can be readily incorporated into the existing examination[71]. Although no studies have directly assessed the relevance of digital chromoendoscopy for the detection of colitis-associated neoplasia and cancer, it has been shown that HD endoscopy with i-scan can detect significantly more neoplastic lesions and more flat adenomas than standard resolution endoscopy[72] and is as precise as dye-based chromoendoscopy for the characterization of small colorectal lesions[73]. Based on these results, data on the assessment of colitis associated dysplasia by digital DLC are eagerly awaited.

Cellular level: Confocal laser endomicroscopy

The technical application of confocal endomicroscopy and the interpretation of images for the utilization in IBD patients can be readily learned. In this regard, it has been shown that after an initial three examinations, performance of CLE significantly improves with a decreased confocal imaging time, successful CLE diagnosis and decline in overall procedural time[74]. In one of the first *in vivo* studies for dysplasia detection, it was shown that using chromoendoscopy (methylene blue) together with endomicroscopy can detect significantly more neoplasia compared to conventional white-light endoscopy while at the same time requiring 50% fewer biopsies[75]. Soon thereafter, CLE was proven to be accurate also for the differentiation between DALM and adenoma-like mass (ALM), thereby facilitating the clinical decision whether patients should receive endoluminal endoscopic resection or be rather referred for proctocolectomy[76]. Importantly, these studies utilized the integrated CLE system (iCLE) and subsequently, another pilot study utilizing probe-based CLE demonstrated that pCLE for dysplasia surveillance in UC is also feasible with reasonable diagnostic accuracy[77] and the typical appearance of DALM on CLE against inflammatory changes has been characterized as dark cells with crypt density attenuation, a ridged-lined irregular epithelial layer with loss of crypts and dilated and distorted vessels with elevated leakage and irregular vascular architecture[42,78]. A recent meta-analysis on the relevance of CLE for dysplasia detection in either patients with sporadic polyps or IBD patients calculated that CLE can distinguish neoplasms from non-neoplastic tissue in IBD patients with a sensitivity of 83% and specificity of 90%, thereby confirming that CLE can indeed differentiate between neoplastic and non-neoplastic tissue[79].

CLE also has been proven to be accurate and efficient for the real-time *in vivo* assessment of mucosal inflammation associated with IBD. One of the earliest pilot studies assessed the morphologic differences on CLE between active and inactive UC and it was shown that colonic crypts in non-active UC are small, round and slightly irregularly arranged with small and round crypt lumina, whereas colonic crypts in active UC appear large, variously shaped, irregularly arranged with numerous inflammatory cells and capillaries in the lamina propria[80]. Soon thereafter, Li *et al*[81] utilized a 4-grade classification of crypt architecture combined with an analysis of microvascular alterations and fluorescein leakage to establish a CLE based classification system for assessment of inflammatory activity in UC patients. All three parameters (crypt architecture, fluorescein leakage, microvasculature) did correlate well with histology, and more than 50% of the patients with normal appearing mucosa on conventional white-light endoscopy exhibited acute inflammation on histology whereas no patient with normal mucosa on CLE showed acute inflammation on histology[81]. Results from our own group indicate that CLE can also reliably assess Crohn's disease activity: a significantly higher proportion of patients with active CD had increased colonic crypt tortuosity, enlarged crypt lumen, microerosions, augmented vascularization, and increased cellular infiltrates within the lamina propria. In quiescent CD, a significant increase in crypt and goblet cell number was detected compared with controls[82] and based on these findings, we proposed the Crohn's Disease Endomicroscopic Activity Score (CDEAS) for the assessment of Crohn's disease activity *in vivo*. The CDEAS does not only allow to differentiate between quiescent CD and controls but also between quiescent and active disease and shows strong correlation to serum levels of the C-reactive protein[82]. Hence, the CLE and the CDEAS are accurate tools for the accurate prediction of disease severity in CD patients[82].

Epithelial gaps, as originally described on CLE by Kiesslich *et al*[83] result from shedding of epithelial cells and are of particular relevance for the endomicroscopic evaluation of inflammatory activity in IBD patients. As shown by Liu *et al*[84], patients with CD exhibit a higher gap density than controls and increased epithelial gap density in the small intestine is a predictor for future hospitalization or surgery in IBD patients[85]. Further, increased cell shedding with fluorescein leakage is

associated with subsequent relapse within 12 mo after confocal examination in IBD patients in remission and a CLE based grading system assessing cell shedding and local barrier dysfunction can predict disease flares with high specificity[86].

Taken together, these results demonstrate that CLE can be used to reliably assess the macro- and microscopic inflammatory activity in IBD patients and to obtain tissue histology in real-time. Since the precise determination of mucosal inflammation is of paramount importance to achieve mucosal healing as a key prognostic parameter and important treatment goal in IBD patients[24], it can be expected that CLE will experience a wider-spread utilization not only to facilitate and optimize the management and surveillance of IBD patients but also to prospectively identify patients that are under risk of experiencing a disease flare.

Cellular level: Endocytoscopy

Compared with CLE, less data on the role of EC for the evaluation of mucosal inflammation in IBD patients are available. In an initial report utilizing an EC system with 450-fold magnification, a newly introduced endocytoscopy score assessing the shape and distance between crypts as well as the visibility of superficial microvessels showed a strong correlation with Matts' histopathological grading[87] and a high reproducibility between different investigators[87]. Recently, our own group tackled the issue whether EC can not only determine inflammatory activity in IBD, but also discriminate single inflammatory cells. For this purpose, we utilized a probe-based EC system with 1390-fold magnification on 19 patients with CD and 21 patients with UC[88]. In this report, we were able to demonstrate that EC is able to reliably distinguish single inflammatory cells, namely neutrophilic, basophilic and eosinophilic granulocytes, and lymphocytes[88]. Further, concordance between endocytoscopy and histopathologic grading of disease activity was 100% and EC exhibited a substantial interobserver and almost perfect intraobserver agreement[88]. The detection of colitis- associated neoplasia or cancer with EC has not been studied to date. However, first evidence suggests that EC can identify dysplasia in aberrant crypt foci as the earliest precursor lesions of colorectal cancer in the dysplasia-carcinoma sequence[89]. In colonic polyps, EC is capable to even detect and distinguish focal high-grade intraepithelial neoplasia[90]. Based on these results, EC is a promising imaging technique that might allow microscopic real-time identification of colitis-associated neoplasia.

Subcellular level: Molecular targeting and molecular imaging

Molecular imaging is based on the utilization of fluorophores with specificity towards a defined molecular target, thereby allowing *in vivo* visualization on the sub-cellular molecular level. Molecular imaging is a rapidly evolving field and with the ongoing identification of crucial molecules involved in the immunopathogenesis of intestinal diseases in basic research, a steadily growing arsenal of targets that can be visualized with molecular imaging becomes available. The ideal probes utilized for molecular imaging in the gastrointestinal tract should exhibit the following characteristics: high diversity, high affinity binding, rapid binding kinetics within minutes, adequate tissue penetration, low immunogenicity, ability for large scale synthesis and florescent labelling[91]. Agents that have been utilized for molecular imaging include the following substance classes: antibodies, lectines, affinity peptides, activatable probes, nanoparticles and physiological substances[92-94]. So far, molecular imaging has been successfully evaluated in mucosal inflammation and cancer development in both, mice and humans. Just recently, Mitsunaga *et al*[95] utilized a topically applied enzymatically activatable probe (gGlu-HMRG) which fluoresces in the presence of γ-glutamyltranspeptidase (GGT), an enzyme associated with cancer, to study colitis-associated cancer detection in a murine model. Using fluorescence colonoscopy in mice, gGlu-HMRG fluorescent lesions were detected 5 min after topical administration, even in small lesions, and fluorescence persisted for at least 30 min. Importantly, at autopsy such lesions corresponded to tumour-containing lesions in all cases analyzed and microscopic inflammatory infiltration exhibited a much lower signal than cancer[95]. Consistent with these observations, others studies successfully detected intestinal dysplasia and polyps in murine and xenograft models *via* the utilization of protease-sensing probes such as a cathepsin reporter probes[96,97], MMP-activatable probes[98], substrates of the γ-glutamylt ranspeptidase[95,99], or certain peptides[100,101]. Apart from that, other studies have chosen molecular targets that are known to be upregulated in colorectal cancer and are already established therapeutic targets such as epidermal growth factor receptor (EGFR) or vascular endothelial growth factor receptor (VEGFR) as fluorescent probes for the detection and precise discrimination of colorectal cancer[102,103]. When targeting VEGFR with fluorescently labeled antibodies, CLE visualized the distribution of VEGF in the malignantly transformed tissue in rodent and xenografted models of colon cancer, as well as in human specimens, and thus allowed identification of cancer cells on subcellular level[102]. For EGFR it was shown that EGFR expression levels of different tumors cell lines in xenograft models could be discriminated *in vivo* in mice with CLE and that topical administration, *i.e.,* the incubation of human colon cancer specimens with the antibody, allowed discerning neoplastic tissue from healthy mucosa[103]. Similar observations were

made in a murine model of gastric cancer[104]. In this study, anti-EGFR antibodies as molecular probes not only successfully identified tumor xenografts but also allowed to visualize the subcellular distribution of EGFR[104]. Based on these results, a first human study was conducted just recently in which the fluorescently-labeled anti-EGFR antibody cetuximab was topically applied in CRC patients[105]. Upon visualization with CLE, an EGFR-specific fluorescence signal was present in 18 out of 19 patients with CRC and 12 out of 18 patients with intestinal adenomas while normal mucosa exhibited no or only weak fluorescence[105].

These findings were directly translated into clinical applications and first pre-clinical trials have impressively demonstrated that the visualization of molecular targeted can be utilized for a risk-stratification of individual patients which allows to predict therapeutic response a priori to the initiation of treatment. One of the first studies that provided proof-of-concept utilized nude mice transplanted with colon cancer xenografts with either high or low EGFR expression[106]. CLE was performed 48 h after injection of a test dose of fluorescently labelled cetuximab and subsequently received cetuximab as a cancer treating agent. Importantly, the CLE-assessed fluorescence intensity before initiation of therapy predicted the response to subsequent cetuximab treatment as shown in a significantly slower tumor progression, better physical condition, and longer overall survival in mice that exhibited tumors with high anti-EGFR fluorescence at the initial evaluation[106].

Just recently it has been shown that molecular imaging with fluorescently labeled antibodies and CLE can successfully be used to stratify IBD patients prior to the initiation of treatment into responders and non-responders, thereby allowing a prediction on the therapeutic success.

In this seminal first phase 1 clinical trial, a fluorescently labeled anti-TNF antibody (FITC-adalimumab) was topically applied to the inflamed mucosa of IBD patients during endoscopy *via* a spraying catheter, and subsequently, the amount of intestinal mTNF+ cells was quantified *via* CLE[107]. Importantly, patients with high numbers of mTNF+ cells showed significantly higher short-term response rates (92%) at week 12 upon subsequent anti-TNF therapy as compared to patients with low amounts of mTNF(+) cells (15%), despite comparable severity of mucosal inflammation in both patient groups. This clinical response in patients with high amounts of intestinal mTNF+ cells was sustained over a follow-up period of 1 year and was associated with mucosal healing observed at follow-up endoscopy[107]. Hence, these data were the first to indicate that molecular imaging with fluorescent antibodies and CLE has the potential to predict therapeutic responses to biological treatment in CD and might be used for personalized medicine in IBD and potentially other autoimmune or inflammatory disorders. The establishment of this approach and its widespread integration into daily endoscopic routine and patient care would have a tremendous impact since it will not only allow to avoid unnecessary risk exposure associated with biological therapies but would also lead to a considerable economization of the treatment regimens.

In summary, as contoured by the studies described above, molecular imaging is a rapidly emerging field in advanced endoscopic imaging and will likely have paradigm-shifting consequences for daily practice in the foreseeable future. With this approach, endoscopy is in the center of attention and allows the endoscopist, apart from diagnosis and treatment, to acquire a third key competence in medicine: prediction on individual patient level.

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TOPIC HIGHLIGHT

2015 Advances in Inflammatory Bowel Disease

Immunogenicity and mechanisms impairing the response to vaccines in inflammatory bowel disease

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Abstract

Inflammatory bowel disease (IBD) is an immunological disorder that is usually treated with immunosuppressive therapy, potentially leading to increases in vulnerability to infections. Although many infections can be prevented by vaccination, vaccination coverage in these patients in clinical practice is insufficient. Therefore, the seroprotection condition should be verified, even for routine vaccines, such as hepatitis B or pneumococcus. Response to vaccines in IBD patients is thought to be impaired due to the immunological alterations generated by the disease and to the immunomodulatory treatments. The immunogenicity of hepatitis B, influenza, and pneumococcal vaccines is impaired in IBD patients, whereas the response to papillomavirus vaccine seems similar to that observed in the healthy population. On the other hand, data on the immunogenicity of tetanus vaccine in IBD patients are conflicting. Studies assessing the response to measles-mumps-rubella, varicella, and herpes zoster vaccines in IBD patients are scarce. The cellular and molecular mechanisms responsible for the impairment of the response to vaccination in IBD patients are poorly understood. Studies aiming to assess the response to vaccines in IBD patients and to identify the mechanisms involved in their immunogenicity are warranted. A better understanding of the immune response, specifically to vaccines, in patients with immune-mediated diseases (such as IBD), is crucial when developing vaccines that trigger more potent immunologic responses.

Key words: Crohn's disease; Inflammatory bowel disease; Tumor necrosis factor; Ulcerative colitis; Vaccine; Vaccination; Immunogenicity

Core tip: Inflammatory bowel disease (IBD) patients

are vulnerable to infections owing to the underlying immunological disorder and to the immunosuppressive therapy used to treat the disease. Although some of these infections could be vaccine-preventable, IBD patients show impaired immunogenicity to some vaccines (such as hepatitis B or pneumococcal vaccines). In this review, the authors discuss available data on the immunogenicity of vaccines in IBD patients and summarize current knowledge on the mechanisms that could impair responses to vaccines.

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INTRODUCTION

Crohn's disease and ulcerative colitis are the two main inflammatory bowel diseases (IBD). Treatment during the last decade has been based on immunosuppressants and biological therapies, such as anti-tumor necrosis factor alpha (TNF) agents[1]. Immunosuppressants and biologics are used increasingly often and earlier during the course of the disease[1]. In this respect, patients with IBD are vulnerable to infections because of the immunological disorder caused by the disease itself or to the immunosuppression induced by the treatments.

Prevention of infectious diseases is a major issue for public health, and vaccination has shown to be one of the most successful strategies against the spread of several diseases. Accordingly, the European Crohn's and Colitis Organisation (ECCO) recommends knowing the seroprotection condition of IBD patients, even for routine vaccines, such as hepatitis B or pneumococcus[2] (Tables 1 and 2). Although numerous groups and experts support the importance of adequate vaccination of IBD patients, the percentage of physicians that monitor and routinely recommend the administration of vaccines to IBD patients is low (approximately 50%)[3-5].

Some studies have suggested that the response to vaccines in IBD patients is impaired[6-10]. The disease-related immune disorder and the immunosuppression induced by the medications could compromise the natural response to immunization and impact the immunogenicity and safety of vaccination in this particular population.

The present review will focus on the immunogenicity of vaccines in patients suffering from IBD and the mechanisms that are potentially involved in impaired response to vaccines.

INACTIVATED VACCINES

Hepatitis B virus vaccination

The prevalence of hepatitis B virus (HBV) infection does not significantly differ between the background population and patients with IBD[11]. However, reactivation of HBV may have fatal consequences in immunosuppressed patients. In this respect, the authors of the REPENTINA 2 study observed that among 25 patients with hepatitis B surface antigen (HBsAg), nine experienced liver dysfunction and six had liver failure[12]. Thus, active preventive measures, such as administration of antiviral drugs, to patients with chronic infection and vaccination of seronegative patients are recommended[2].

Recombinant HBV vaccines mainly consist of HBsAg associated with adjuvants that enhance the immune response (*e.g.*, monophosphoryl lipid A, aluminium hydroxide, oil-in-water emulsions). Studies in healthy individuals showed that three doses of HBV vaccine were enough to develop protective anti-HBs antibody titers in over 95% of the population[13-15]. However, the immunogenicity of this vaccine in IBD patients has proven to be lower, mainly in those patients receiving biologic therapy or immunosuppressants[16,17]. For example, Melmed *et al*[18] detected anti-HBs antibodies in only three out of nine patients, and Vida Pérez *et al*[19] in 36% of the vaccinees. In another study with a single-dose vaccine at 0, 1, and 6 mo, an appropriate immune response (*i.e.*, > 10 IU/L) was obtained in all healthy controls, but only 76% of patients were able to reach that cutoff[6].

The largest study to date on HBV vaccination in IBD patients was performed by Gisbert *et al*[20]. A total of 241 patients were vaccinated against HBV with a quick schedule (0, 1, and 2 mo) and a double-dose protocol. Fifty-nine percent and 39% of the patients developed, respectively, anti-HBs titers > 10 IU/L and > 100 IU/L two months after the last dose. In this study, older age and anti-TNF treatment were associated with a lower response rate.

These findings were confirmed by Loras *et al*[21], who studied 254 patients (235 with anti-HBs < 10 IU/L and 19 with anti-HBs from 10 to 100 IU/L). In this study, only 26% of patients achieved anti-HBs titers > 100 IU/L. Age ⩽ 30 years and starting the vaccination schedule simultaneously with anti-TNF treatment (*vs* months to several years of anti-TNF treatment) were the only predictors of effective vaccination.

The second ECCO consensus on opportunistic infections suggested that the development of sero-protection might require higher doses of the VHB immunogen[2]. The benefit for vaccinating with a high-dose protocol was demonstrated by Gisbert *et al*[22], who studied 148 patients vaccinated against HBV using two different protocols: 54% with the "clinical

Table 1 Vaccines recommended in patients with inflammatory bowel disease

Vaccine	Type of immunogen	General recommendations for vaccination in IBD	Concerns in IBD patients on immunosuppressive therapy
HBV	Recombinant protein	After checking the serological status for HBV: double-dose schedule	None
HPV[1]	Quadrivalent vaccine (Recombinant proteins)	Women aged between 11-12 yr: 3 doses (0, 2 and 6 mo)	None
Influenza	Inactivated virus	1 dose annually	None
Pneumococcus	Polysaccharides, conju-gated or not to a protein carrier	1 dose every 5 yr	None
Tetanus	Inactivated toxoid	Patient previously vaccinated: 1 dose every 10 years Unknown or not previously vaccinated: 3-doses	None
Measles-mumps-rubella	Live attenuated virus	Non-immunized: Standard schedule	Contraindicated
Varicella	Live attenuated virus	Non-immunized: 2 doses (0 and 1-2 mo)	Risks and benefits should be evaluated on an individual basis
Herpes zoster[1]	Live attenuated virus	Patients aged over 60 yr: Standard schedule	Risks and benefits should be evaluated on an individual basis

[1]Depending on local recommendations. Source: Second European evidence-based consensus on the prevention, diagnosis and management of opportunistic infections in inflammatory bowel disease[2]. IBD: Inflammatory bowel disease; HBV: Hepatitis B virus; HPV: Human papillomavirus.

Table 2 Vaccines recommended in patients with inflammatory bowel disease and mechanisms associated with impaired response in these patients

Vaccine	Immunogen	Impaired response	Factors associated with a lower response	Mechanisms associated with lower immunogenicity
HBV	Recombinant protein	Yes	Age[20,21,23], immunosuppressive or anti-TNF therapy[20,21,23]	Not described
HPV	Recombinant protein	No	-	-
Influenza	Inactivated virus	Yes	Immunosuppressive therapy[8]	Not described
Pneumococcus	Polysaccharides	Yes	Immunosuppressive and/or anti-TNF therapy[9,10,55]	Conflicting results about memory B cells[60,62]
Tetanus	Inactivated toxoid	Unclear	None described	Defects in the development of IgG-secreting plasma cells[65]
Measles-mump-rubella	Live attenuated virus	No	-	-
Varicella	Live attenuated virus	No	-	-
Herpes zoster	Live attenuated virus	No	-	-

HBV: Hepatitis B virus; HPV: Human papillomavirus; TNF: Tumor necrosis factor.

practice" protocol (single doses of Engerix-B® at 0, 1, and 6 mo) and 46% with a faster, double-dose protocol (double doses of Engerix-B® at 0, 1, and 2 mo). A higher effective response to vaccination (defined as anti-HBs > 10 IU/L) was reached with the faster double-dose schedule than the response obtained with the single-dose protocol (75% *vs* 41%). The double-dose protocol was the only factor associated with a better response to the vaccines, suggesting that the faster double-dose schedule could be a suitable option in patients with IBD[22].

Although the double-dose regimen was more immunogenic than the standard dose, the response to HBV vaccine in IBD patients was still too low compared to healthy controls. Chaparro *et al*[23] assessed the immunogenicity of a recombinant vaccine with a new adjuvant, Fendrix®, compared with double-dose Engerix® at 0, 1, 2, and 6 mo in IBD patients. A four-dose vaccine schedule significantly increased (by > 40%) the response compared with the three-dose regimen. Older age and treatment with immunosuppressants or anti-TNF drugs impaired the success of the vaccines.

Therefore, despite the numerous attempts to enhance the response to HBV vaccines either by increasing the dosage, optimizing the administration schedule, or testing potent new adjuvants, the response rate to HBV vaccine in IBD patients was still impaired.

The success of the recombinant HBV vaccine depends mainly on the T-cell response to the antigen. However, before such a response can occur, antigen-presenting cells must be able to present the antigen to the T cells, and B cells must be able to proliferate and differentiate into anti-HBs-secreting plasma cells. Thus, the development of protection against HBV will largely depend on the ability of the immune system to produce anti-HBs antibodies. Nevertheless, long-term protection against infection may also require generation of immune memory cells (B and T memory

lymphocytes)[24].

The response to HBV vaccine does not only depend on the type and dosage of HBV vaccine. Vaccinee characteristics, such as age, gender, the presence of certain genetic polymorphisms, comorbidity, immune status, or smoking habit, also affect the immunogenicity of the HBV vaccine[25].

Many studies have investigated the immune mechanisms associated with the responsiveness to HBV vaccine in the healthy population. For example, an association between human leukocyte antigen (HLA) haplotypes and defects in the presentation of HBsAg (by antigen-presenting cells) and recognition HBsAg (by T lymphocytes, affecting their cytokine production profile) has been described[26]. The role of lymphocytes in triggering the immune response has also been investigated, and defects in the lymphocyte repertoire or functionality have been documented[27-29], as has the presence of T-cell populations that suppress the cellular response to HBsAg[30] and abnormal regulatory T-cell counts[31]. Finally, diminished activation of natural killer (NK) and natural killer T (NKT) cells has also been associated with a poorer response to this vaccine[32].

Immune-mediated or chronic viral diseases, such as human immunodeficiency virus (HIV) infection and chronic liver or kidney disease, have also been associated with impaired responsiveness to the HBV vaccine. For example, it has been suggested that one of the main reasons for vaccine failure in patients with chronic viral infections [HIV, hepatitis C virus (HCV)] is the limited proliferative potential of the lymphocyte associated with changes (induced by the infective virus) in the signaling immune mechanisms[33]. Furthermore, an impaired T-helper response has been reported in patients on dialysis[34]. On the other hand, biological parameters, such as higher helper T-CD4 prevaccination counts in HIV-infected patients[35] or a higher CD4/CD8 ratio in dialysis patients[36], have been shown to predict a better response to vaccination.

In IBD patients, data on the cellular or molecular mechanisms impairing the immunogenicity of HBV vaccine are scarce. Several of the genetic mutations and polymorphisms associated with an increased risk of developing IBD have also been involved in recognition of intestinal microbiota by the innate immune system (*NOD2*, *TLR4*), in autophagy (*ATG16L1*, *IRGM*, *VAMP3*), in intestinal barrier function (*DLG5*, *MUC1*), and in the activation, survival, and growth of lymphocytes (*HLA*, *IL23R*, *IL10*, *IL10R*, *IL2RA*, *ERAP2*, *CPEB4*, *TNFSF11*, *SMAD3*)[37,38]. The genetic and immunological peculiarities of patients with IBD described above, together with the effect of the immunomodulatory therapies, could, therefore, affect the ability of the immune system to react properly to the vaccine antigens.

Human papillomavirus vaccination

Human papillomavirus (HPV) infection is a sexually transmitted disease that comprises some 40 oncogenic variants classed as low to high-risk to develop an anogenical neoplasm[39-41]. As HPV-associated tumors may be more common after prolonged immunosuppressive therapy[2], vaccination has been recommended in patients with HPV infection[41].

Since 2006, a quadrivalent vaccine that covers types HPV-6, -11, -16, and -18, is accessible in Europe. In 2007, a bivalent vaccine for types HPV-16 and -18 was authorized. Both prophylactic vaccines are effective and safe against HPV in the immunocompetent population (95%-100%)[42,43].

Jacobson *et al*[44] assessed the immunogenicity and tolerability of the quadrivalent HPV vaccine in IBD patients receiving immunosuppressive therapies and in healthy controls. The study included 33 IBD patients who received three doses of Gardasil® at 0, 2, and 6 mo. After the three doses, 94% of the patients seroconverted to the four subtypes of HPV, and only 6% were not seropositive to type HPV-18. This figure was similar to that described in healthy individuals. Unfortunately, owing to the small sample size, the study did not provide data on differences in immunogenicity between the different drug doses (immunomodulators *vs* anti-TNF agents).

Influenza virus vaccination

Influenza is a seasonal respiratory disease that, despite its usual acute and self-limiting behaviour, leads to many thousands of visits to emergency departments and can be lethal[8,45,46]. Rates of morbidity and complications have been reported to be higher among immunosuppressed patients[47,48].

The A and B types of the virus are responsible for human influenza epidemics. Immunosuppression increases the risk of infection, and, therefore, the annual vaccination for patients on immunosuppressants has been proposed[2]. Whereas the majority of patients suffering IBD will receive immunosuppressive therapy during the course of their disease, the ECCO consensus recommends annual vaccination since the disease was diagnosed[2].

There is a live-attenuated influenza vaccine and also an inactivated type. The live-attenuated one is not recommended for patients on immunomodulators, but the trivalent inactivated influenza vaccine is not contraindicated in patients on immunosuppressants[49].

Influenza vaccine seems to be less immunogenic in IBD patients, especially evidenced by a low serologic responses against the virus type B[7,8,50,51]. For example, Mamula *et al*[7] included 51 children with IBD and 29 healthy controls and found a significantly poorer immune response in IBD patients than in healthy controls. Furthermore, patients receiving infliximab and immunomodulators were less likely to respond to influenza vaccine antigens. These results were also confirmed by deBruyn *et al*[8] in a study that included 60 children with IBD and 53 healthy controls

who received inactivated influenza vaccines, including both type A (H1N1 and H3N2) and type B. In this study, children with IBD showed a diminished response to the B component (53%) compared to healthy individuals (81%).

The negative effect of immunosuppression on the response to the influenza vaccine has been assessed in several diseases. For example, Cowan *et al*[52] observed lower immunogenicity of the vaccine in immunosuppressed kidney recipients than in healthy people. This diminished response seemed to be associated with a defective humoral and cellular response and with suppression of differentiation of B cells into IgG-secreting plasma cells supported by immunosuppressive therapy. A recent study by Bálint *et al*[53] showed that the administration of the vaccine in IBD patients (74% of whom were receiving immunosuppressive therapy) induced a decrease in serum IL-2 levels. Other immune-mediated and chronic viral diseases, such as rheumatoid arthritis, HIV, and common variable immunodeficiency, have been associated with an impaired immune response to the influenza vaccine, thus highlighting the importance of vaccinee immune status.

Genetic polymorphisms have also been associated with the response to influenza vaccination[54].

In conclusion, despite the fact that the response to influenza vaccine appears to be diminished in IBD patients taking immunosuppressant drugs, the degree of response reached in most cases seems to be enough, so the annual influenza vaccination is recommended[12].

Pneumococcal vaccination

Streptococcus pneumoniae is a pathological micro-organism that is able to cause serious infections, such as meningitis or pneumonia. Cohort studies have shown that one of the most prevalent infections in immunosuppressed patients with IBD is bacterial pneumonia[55], maintaining these patients at high risk of invasive pneumococcal disease[16,18]. Accordingly, it is recommended to administer, at least, one dose of the pneumococcal vaccine to all IBD patients[2].

Two types of pneumococcal vaccine are available: the 23-valent polysaccharide vaccine and the conjugate vaccines (polysaccharides conjugated to proteins, such as diphtheria and tetanus toxoids, meningococcal outer membrane protein complex or protein D of *Haemophilus influenzae*). Both types of vaccines can be used in IBD patients, but most studies have focused on the 23-valent polysaccharide vaccine.

The immunogenicity of pneumococcal polysaccharide vaccination has been assessed in IBD patients. Study results suggested that IBD patients receiving immunosuppressants have significantly impaired postvaccination titers, while not immunosuppressed patients and healthy people do not and have similar response rates to one another. Moreover, patients on combination therapy (*i.e.*, taking more than one immunosuppressant) had a lesser immune response to the pneumococcal vaccine than patients treated with only one immunosuppressive drug in monotherapy[9,10,55]. As these data reflect that, somehow, immunosuppressant therapy influences the outcome of the 23-valent pneumococcal vaccine, it is advisable to administer the vaccine at diagnosis or at least 2 wk before starting any immunomodulatory treatment[13,55]. A booster dose should be administered after 5 years[2]. Despite the suboptimal response to vaccination among IBD patients receiving immunomodulators or biological drugs, the vaccine could still confer some degree of protection[3].

Pneumococcal 23-valent vaccine is composed of polysaccharides that are T-cell-independent antigens, which do not induce immunologic memory. B lymphocytes are responsible for recognizing polysaccharides and secreting protective antibodies against pneumococcal bacteria (IgG and IgM). The phenotype of the B cells that react specifically against the 23-valent vaccine has not been fully identified, although, at least in young healthy people, most seem to be IgM^+ memory B cells[56]. In elderly people, however, the response to the 23-valent vaccine was mediated by switched memory B cells (IgM^-) instead of IgM^+ memory B cells[57]. This "alternative" immunological mechanism that generates protection through switched memory B cells was also associated with decreased opsonophagocytic activity[57]. People with low counts of IgM^+ memory B cells (*e.g.*, the elderly or patients with common variable immunodeficiency) showed diminished efficacy of pneumococcal vaccine and increased susceptibility to infections caused by encapsulated bacteria, such as *S. pneumoniae*[58,59]. Notably, IBD patients, even those who are not receiving immunomodulators, also have a lower proportion of circulating IgM^+ memory B cells than healthy controls, probably owing to deficient spleen function[60,61].

Other studies that have investigated the relevance of switched memory B cells in IBD patients have shown conflicting results. Di Sabatino *et al*[60] compared the percentage of circulating switched memory B cells between patients with IBD and healthy adults and found no significant differences. In contrast, Fallahi *et al*[62] found fewer switched memory B cells in children with Crohn's disease (but not in those with ulcerative colitis) than in healthy young adults vaccinated with a nonconjugate pneumococcal vaccine.

An increase in the proportion of IgM^+ memory B cells has been observed in IBD patients who respond to anti-TNF drugs[63]. This finding has been confirmed in patients with spondyloarthritis receiving anti-TNF therapy[64]. To the best of our knowledge, no study has assessed the possible relationship between switched/unswitched memory B-cell counts, opsonization activity, use of immunosuppressants, and response to pneumococcal vaccine in IBD patients.

In contrast to vaccines that include only poly-

saccharides, conjugate pneumococcal vaccines have the advantage of inducing both humoral response and immune memory. However, despite their potential benefits in IBD patients, conjugate pneumococcal vaccines have been poorly studied.

Tetanus

Patients with IBD not vaccinated against tetanus or with unknown vaccination status should receive the primary series of tetanus vaccines (three doses). After the initial series, all patients should receive the booster every 10 years. Three studies have investigated the serological response to the booster vaccine in IBD patients and found conflicting results: two studies suggested an altered response[65,66], while the third observed normal anti-tetanus antibody titers[67]. Brogan *et al*[65] suggested that the impaired response to the tetanus vaccine in IBD patients could be caused by a defect in the development of IgG-secreting plasma cells; however, this finding has not been confirmed elsewhere.

LIVE-ATTENUATED VACCINES

Measles, mumps and rubella

Since the vaccine against measles, mumps, and rubella is commonly administered in childhood, it is usually given before IBD is diagnosed. Vaccine can be administered in IBD patients not treated with immunosuppressant drugs and lacking immunity. Nevertheless, as this vaccine is generally given in most developed countries, the risk of acquisition of these infections is very low[68].

Varicella and herpes zoster vaccinations

Varicella infection is generally a mild disease in children, but it can develop severe complications, especially in adults, leading to death in 20/100000 people[69]. Immunity to varicella is usually acquired through infection during childhood[18]; however, as this illness is very contagious, adults not immunized are at high risk of be infected. Since a third of infected immunocompromised patients have a disseminated herpes zoster disease[69], it is recommended to confirm the seroprotection of IBD patients before the administration of an immunomodulator.

Local guidelines generally recommend the vaccination of children between the ages of 12 and 18 mo and administration of a booster dose at 11-12 years. Children with IBD not treated with immunosuppressant drugs should follow the same vaccination protocol[2]. In the case of adult patients with IBD not immunized against varicella, it is recommended to administer the two-dose series of varicella vaccine at least 3 wk before starting any immunomodulatory therapy[2]. Although recent studies show that this vaccine is effective and safe, even in immunosuppressed patients, data are still scarce. Given the potential risk of complications due to the progression of the infection in immunocompromised adults, the benefits and risks of the varicella vaccine should be considered on an individual basis.

After resolution of the varicella infection, the virus stays latent within the spinal ganglion. The reactivation of the virus results in the Herpes zoster infection (shingles), that is developed in up to one in three people in the general population and in an higher rate among immunocompromised patients[69].

A herpes zoster vaccine has been licensed in the United States. This vaccine is a live-attenuated strain of the varicella zoster virus, 14 times more potent than the single-antigen varicella vaccine, and it is suggested for people over 60 years in order to prevent and/or reduce the severity of herpes zoster complications[70]. As little information is available regarding the safety and efficacy of the vaccine in immunocompromised patients, and immunosuppression can lead to a disseminated disease in case of infection, guidelines do not recommend the administration of the shingles vaccine in patients treated with anti-TNF drugs[71] and suggest a window of 1-3 mo after initiating immunosuppressive therapy[72-74]. Nevertheless, the Centers for Disease Control (CDC) and the Advisory Committee on immunization Practices (ACIP) stated that patients with lower levels of immunosuppression ($\leqslant$ 0.4 mg/kg per week of methotrexate, $\leqslant$ 3 mg/kg per day of azathioprine, or $\leqslant$ 1.5 mg/kg per day of mercaptopurine) can tolerate attenuated herpes zoster-based vaccine. In fact, the risk of recurrence of varicella is low, even in profoundly immunosuppressed patients, as varicella-zoster immunity is well-maintained over time[71].

In this respect, Zhang *et al*[72] studied the incidence of herpes zoster disease after administering the live-attenuated vaccine in a cohort of 450000 patients with immune-mediated diseases (including IBD). The study concluded that the short-term risk of herpes zoster was not increased in vaccinated patients, independently of the prescription of anti-TNF therapy. Moreover, a decline in the incidence of herpes zoster over a median 2 years of follow-up was related to the vaccination[72]. However, the proportion of vaccinated patients was small (1.2%), suggesting that further evidence is needed to confirm the safety of the vaccine in this population.

CONCLUSION

Patients with IBD are at risk of vaccine-preventable illnesses. The immunization status of patients with IBD should be verified, even with respect to routinely administered vaccines. It has been suggested that the response to vaccines in IBD patients is impaired owing to the immunological alterations generated by the disease and to the immunomodulatory treatments. The immunogenicity of hepatitis B, influenza, and pneumococcal vaccines is impaired in IBD patients,

whereas the response to papillomavirus vaccine seems to be similar to that observed in the healthy population. Data on the immunogenicity of tetanus vaccine in patients with IBD are conflicting. Studies assessing the response of patients with IBD to measles-mumps-rubella, varicella, and herpes zoster vaccines are scarce. The mechanisms involved in the altered response to vaccines in IBD patients remain unclear. Several HLA haplotypes have been associated with a higher risk of vaccination failure; however, whether these genetic factors cause deficient antigen presentation or diminished recognition by immune cells remains unknown.

Studies aiming to assess the response to vaccines in IBD patients and to identify the mechanisms involved in their immunogenicity are warranted. Understanding the alterations of the immune system of IBD patients is a key area in the development of more immunogenic vaccines for this particular group of patients and for other patients with immune-mediated diseases.

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TOPIC HIGHLIGHT

2015 Advances in Inflammatory Bowel Disease

Current stage in inflammatory bowel disease: What is next?

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Abstract

In recent years, the incidence of inflammatory bowel disease (IBD) has been on the rise, extending to countries where it was infrequent in the past. As a result, the gap between high and low incidence countries is decreasing. The disease, therefore, has an important economic impact on the healthcare system. Advances in recent years in pharmacogenetics and clinical pharmacology have allowed for the development of treatment strategies adjusted to the patient profile. Concurrently, new drugs aimed at inflammatory targets have been developed that may expand future treatment options. This review examines advances in the optimization of existing drug treatments and the development of novel treatment options for IBD.

Key words: Inflammatory bowel disease; Future directions; Pharmacogenetic; Pharmacokinetics; New drugs

Core tip: The incidence and prevalence of inflammatory bowel disease (IBD) has been increasing worldwide. In recent years, the treatment objectives, the monitoring of IBD, and the drug treatments for controlling the disorder have been evolving. This review summarizes recent developments in pharmacogenetics, clinical pharmacology, and the use of new drug molecules that may expand IBD treatment options in the future.

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INTRODUCTION

Inflammatory bowel disease (IBD), Crohn's disease (CD), and ulcerative colitis (UC) are important public health problems. According to recent studies, the annual incidence of UC varies between 19.2-24.3 cases per 100000 inhabitants in Europe and 6.3 cases

per 100000 inhabitants in Asia and the Middle East[1]. For CD, the estimated incidence is 12.7-20.2 cases per 100000 inhabitants in Europe and the United States *vs* five cases per 100000 inhabitants in Asia and the Middle East. The incidence of IBD is currently growing in areas where the disease was previously infrequent. As a result, the gap between high- and low-incidence countries is closing[2]. This rise runs parallel to technological development, improvements in living standards, and a greater interest in this disease among physicians[3]. The underlying pathogenesis remains uncertain, although the most widely accepted theory revolves around changes in the host immune response in genetically susceptible individuals to the intestinal microbiota that is triggered by environmental stimuli. None of these alterations alone can cause the disease, and the interactions among these four factors in the pathogenesis are very complex. In recent decades there have been important advances regarding each of these factors. Progress in the field of genetics has resulted from the performance of genome-wide association studies (GWAS), although they only account for 20%-25% of the cases of IBD[4]. Knowledge of epigenetic mechanisms could explain the influence of environmental factors and the microbiota upon IBD and the low correlation to concrete genes[5,6]. These developments have opened the door to personalized medicine[7].

Knowledge of the immunological mechanisms involved in the manifestation of IBD has led to the development of new biological drugs. The first major advance is represented by the anti-tumor necrosis factor (TNF)-α drugs, which have revolutionized the treatment of IBD, since they are able to induce and maintain mucosal healing of the disease[8], a key factor for modifying the natural course of the disorder[9,10]. Nevertheless, despite these advances, one-third of all patients with CD fail to respond to anti-TNF-α therapy (primary non-responders), and 10% do not tolerate or do not respond to any of the drugs used to treat CD[11,12]. In the case of UC, the reported colectomy rate reaches up to 21% after an initial response to anti-TNF-α drugs[13]. This has led to the search for new therapeutic targets and further optimization of existing treatment options. Clinical pharmacology allows us to determine therapeutic drug concentrations (thiopurine agents and anti-TNF-α drugs) and, if needed, to explain their loss of responsiveness and their adverse effects. In the coming years, personalized medicine, where treatments will be prescribed according to the risk factors in each individual patient and the probability of achieving response to a given drug substance, will be initiated. There have been developments in the way IBD is monitored, with the adoption of reliable and scantly aggressive techniques, such as noninvasive imaging tests, stool markers, breath tests, *etc*.[14], which fall beyond the scope of this review. We provide below a description of the current advances in pharmacogenetics and possible new drug substances.

PHARMACOGENETICS

Personalized medicine seeks to find the ideal drug for each individual patient at the appropriate dose and administered *via* the best possible route. This approach allows for increased effectiveness, with the least risk of side effects, and at the lowest possible cost. Physicians try to identify patients with more serious disease, with a view to introducing early and more effective treatment in order to prevent long-term complications, distinguishing them from those individuals with less severe disease and a more favorable prognosis in which aggressive treatment poses a higher risk of undesired effects. Patient response to drug treatment is dependent upon many factors, including the severity of the disease and genetic and environmental factors.

Pharmacogenetics is the study of the association between the different polymorphisms of a gene and the variability of response to treatment or its toxicity with a given drug. It has been estimated that polymorphisms can account for 20%-95% of the variability of a response to a drug[15].

A number of drugs are currently available for the treatment of IBD: 5-aminosalicylates, corticosteroids, immunosuppressors (thiopurine drugs, calcineurinic agents, methotrexate), and biological agents (anti-TNF-α drugs).

Aminosalicylates

The aminosalicylates are among the main agents used to treat patients with UC, and their colon cancer chemoprophylactic effect allows them to be used in UC with pancolonic disease involvement. The metabolization of both sulfasalazine and mesalazine is mediated by the enzyme N-acetyltransferase (NAT). For almost six decades, the population has been divided into fast and slow acetylators. There are two NAT isoenzymes (NAT1 and NAT2), and different polymorphisms have been described in different ethnic groups[16]. NAT1 metabolizes mesalazine, and it has no demonstrable associations with clinical effects. NAT2 metabolizes salazopyrin derived from sulfasalazine breakdown. In 1983, a link between NAT2 slow acetylators, who accumulate higher drug levels in blood, and an increased number of side effects was shown. Twenty-five years later, and thanks to our knowledge of single nucleotide polymorphisms (SNPs), it has been possible to confirm the association between NAT2 with a slow acetylator phenotype and dose-dependent side effects[17]. There are fewer studies on 5-acetylsalicylic acid (5-ASA) than with immunosuppressors and biological drugs, since 5-ASA is only used to reduce side effects that are usually not serious. However, since more prolonged treatment with 5-ASA was proposed due to its chemoprotective effect against colon cancer, the pharmacogenetic studies have become more important.

Glucocorticoids

Glucocorticoids (GLCs) are used in moderate and severe flare-ups of IBD, and although they are very effective, 16%-20% of all patients are refractory to GLCs in the Caucasian population, and 28%-36% are corticodependent[18-20]. The GLCs exert their anti-inflammatory effect by inhibiting T cell activation and cytokine secretion, following binding of the drug to the intracellular glucocorticoid receptors (GR-alpha), which modify their structural conformation as a result. Three potential mechanisms can cause GLCS treatment to be ineffective: inadequate receptor function; an excess of proinflammatory cytokines, which would reduce affinity between the drug and its intracellular receptor; and a decrease in intracellular corticosteroids secondary to expulsion from the cell[21]. This latter mechanism is dependent upon glycoprotein P-170 (P-gp), which is found in lymphocytes and in the apical membrane of the enterocytes, among other locations. An increase in P-gp at cell surface level causes drug release into the bloodstream. This protein is encoded by the ABCB1/MDR1 gene of chromosome 7. The expression of this gene is reportedly increased in IBD presenting a greater need for surgery because of a poor response to drug treatment[22]. Different allelic variants (the most widely studied being C3435T and G2677T) are associated with an increased risk of developing extensive UC, although no association to CD has been observed[23]. Studies with larger patient series and stable corticosteroid doses are needed to determine the precise relationship between P-gp and the lack of response to such drugs.

The studies that have explored the different cytokines implicated in corticosteroid response offer contradictory results, and the underlying polymorphisms have not been established[24].

Genetic studies related to the gene encoding for the intracellular glucocorticoid receptor (*hGR*) have also been performed. Polymorphism N363S is associated with a good response[25], while polymorphism ER22/23EK is associated with corticosteroid resistance[26]. Knowing the genetic susceptibility of corticosteroid resistant patients is an important step forward, since it would help avoid important morbidity among patients who stand to derive no benefit from such treatment. None of these pharmacogenetic markers are of use in routine clinical practice.

Thiopurine drugs

Thiopurine drugs are used to maintain remission in patients with moderate to severe IBD. The effects are only observed after 3 mo of treatment. Purine metabolization is complex and involves different enzymes; this results in important genetic variability in the efficacy and toxicity of these drug substances (Figure 1).

The thiopurine drugs (TPs) are able to control the disease in 66% of patients, although an important proportion (10%-25%) must suspend the medication because of serious (leukopenia, pancreatitis, infections, or malignancies) or mild side effects (rash, nausea, vomiting, flu syndrome, or joint pain). A clear association has been demonstrated between thiopurine methyltransferase (TMPT) deficiency and bone marrow suppression, although this explains only one-third of adverse effects. No clear explanation has been found for the remaining effects. Knowing in advance whether these drugs will be tolerated and the risk of side effects in a given patient may prove useful in daily practice.

TPMT: TPMT is the most widely studied enzyme in IBD. The identification of genetic mutations before onset of treatment with TPs is currently the only pharmacogenetic test performed in IBD.

In 1980, Weinshilboum and Sladeck were the first to describe the trimodal distribution of TMPT in Caucasian patients: 90% of the subjects have normal TMPT activity, almost 10% have intermediate activity, and 0.3% have almost zero activity. Posteriorly, over 30 allelic variants have been described, with different distributions depending on the ethnic group considered. The correlation between genotype and phenotype (expressed enzyme activity) is very good in 77%-99% of the cases. The differences can be explained by genetic and epigenetic factors (such as the use of concomitant drugs that inhibit TMPT), the age of the patient, and the existence of recent transfusions - since two different enzyme populations (donor and recipient) may be measured[27,28]. The genetic study of this enzyme allows us to distinguish among homozygous individuals (without enzyme activity) at a high risk of suffering bone marrow suppression; ultra-fast methylators (high enzyme activity) with high liver toxicity and a low response to treatment; and patients with normal and intermediate enzyme activity, which are the individuals that stand to benefit most from this medication and are at lesser risk of adverse effects. Furthermore, it can help determine which dose a treatment should be started (Table 1).

Allopurinol reduces the levels of 6-methylmercaptopurin (6-MeMp) and increases those of 6-thioguanine (6TG). Although its mechanism of action is not clear, it has been suggested that the drug inhibits the enzyme xanthine oxidase through competitive inhibition or reduces the availability of its substrate[29,30]. In daily practice, the TP dose should be reduced by 25% in those patients who require allopurinol and present with a normal TPMT genotype (Table 1). Taking into account that only about one-third of all cases of bone marrow suppression in patients receiving TPs are explained by genetic disposition and that the origin is, therefore, multifactorial and will require constant laboratory test monitoring, many authors have questioned whether this strategy is cost-effective. Nevertheless, most current clinical

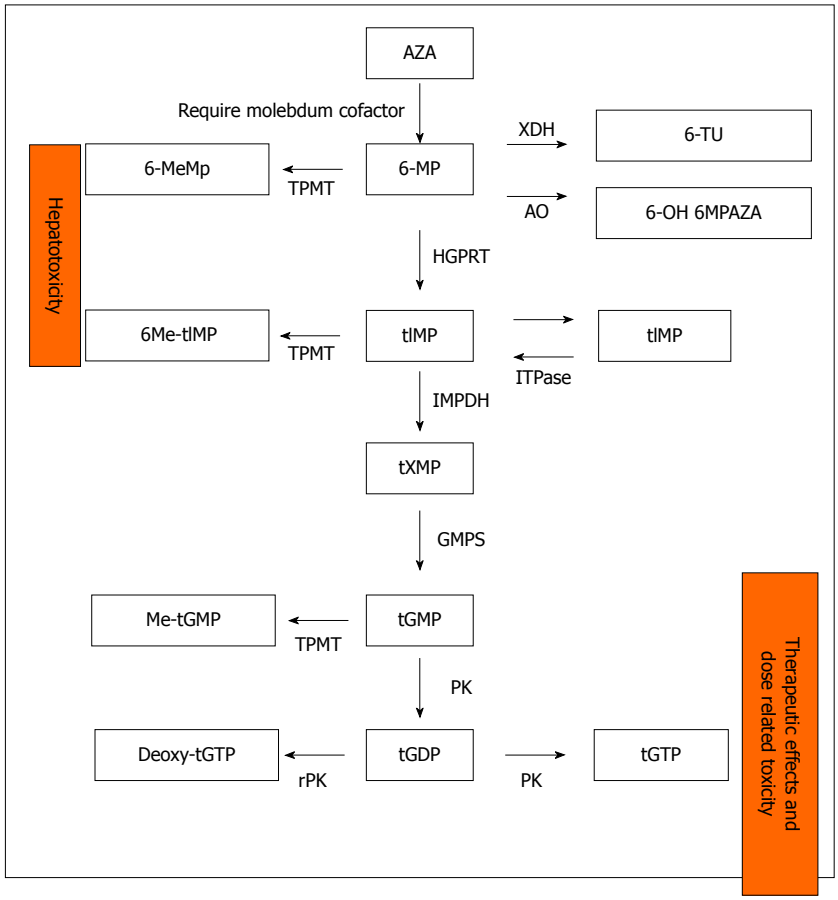

Figure 1 Metabolism of azatioprine and 6-mercaptopurine. AZA: Azathioprine; 6-MP: 6-mercaptopurine; 6-MeMP: 6-methylmercaptopurine; 6-TU: 6-thiouric acid; 8-OH 6MP: 8-hydroxymercaptopurine; 6Me-tIMP: 6-methyl thioinosine monophosphate; 6-tIMP: 6-thioinosine monophosphate; 6-TIDP: 6-thioinosine diphosphate; 6-TITP: 6-thioinosine triphosphate; 6-MeTITP: 6-methylthioinosine triphosphate; tXMP: Thioxanthine monophosphate tGMP: Thioguanine monophosphate; tGDP: Thioguanine diphosphate; tGTP: Thioguanine triphosphate; Me-tGMP: Methylthioguanine monophosphate; Deoxy-tGTP: Deoxythioguanine triphosphate; GST: Glutathione S-transferasa; TPMT: Thipurine methyltransferase; XDH: Xanthine dehydrogenase; AO: Aldehyde oxidase; HGPRT: Hypoxanthine guanine phosphoribosyltransferase; IMPDH: Inopine monophosphate dehydrogenase; GMPS: Guanosine monophosphate synthetase; PK: Phosphokinase; rPK: Reductase phosphokinase.

Table 1 Azatioprine treatment basis of individual thiopurine methyltransferase status

TMPT genotype	TPMT fenotype (pmol/mgHb)	Treatment dosis (mg/kg)
Homozygous	< 10	Avoid or consider 0.1-0.2
Heterozygous	10-24	1-1.5
Wild type (normal)	25-35	2-2.5
Wild type (high)	> 35	0.5 + 100 mg allopurinol

TMPT: Thiopurine methyltransferase.

guides do not recommend genetic study before starting treatment with TPs. On the other hand, such studies are not available in many hospitals; laboratory test monitoring is, therefore, the safest alternative.

Less scientific evidence is available regarding the other enzymes.

The enzyme xanthine oxidase (XO) is the second most important enzyme in the metabolism of TPs. It is found in many tissues, although its main activity is located in the small bowel and liver. Until a few years ago, it was only known that XO activity varied from one person to another. Discordant variations depending on patient gender or ethnicity had been described[31,32], together with changes induced by environmental factors, such as smoking and the diet. In 2008, a Japanese group described three polymorphisms of the gene encoding for XO (G514A, A3326C and A3662G) that are linked with enzyme activity, with the population being divided into low, normal, or high activity[33]. These polymorphisms have not been studied in Caucasians or in patients with IBD. At present, we are only able to extrapolate that individuals with low enzyme activity would be at greater risk of suffering adverse effects, while those with high activity would experience treatment failure.

The enzyme inosine triphosphate pyrophosphatase (ITPase) controls the intracellular levels of inosine triphosphate (ITP), transforming it into inosine monophosphate, which acts as a substrate for other enzymes. When the enzyme is deficient, ITP accumulates in enterocytes. Deficiencies of this enzyme have been known for almost half a century and

Table 2 Genetic association studies of adverse events secondary to azathioprine in inflammatory bowel disease patients

Study (year)	No. of patients	Conclusion
Marinaki *et al*[34] (2004)	130	Significant association with flu-illness, rash, and pancreatitis No association with mielotoxicity
Allorge *et al*[35] (2005)	72	No association with flu-illness, rash, pancreatitis, or mielotoxicity
Gearry *et al*[36] (2004)	147	No association with flu-illness, rash, and pancreatitis
De Ridder *et al*[37] (2006)	72	No association with side effects
Hindorf *et al*[38] (2006)	60	No association with side effects
von Ahsen *et al*[39] (2005)	71	Early withdrawal of therapy but no association with specific adverse events
Ansari *et al*[40] (2008)	202	Association with flu-like symptoms but not withdrawal of therapy
van Dieren *et al*[41] (2005)	109	Not associated with an increased risk for the development of leucopenia and other side effects
Zelinkova *et al*[42] (2006)	262	Increased risk of leucopenia
Uchiyama *et al*[43] (2009)	16	Increase risk of mielotoxicity (leucopenia)
Shipkova *et al*[44] (2011)	160	Increase risk of mielotoxicity
Kim *et al*[45] (2010)	248	No association with leucopenia
Zabala-Fernández *et al*[46] (2011)	232	Significant association with artralgia

have been evaluated in different ethnic groups (with incidences of 5%-7% among Caucasians and African populations and up to 15% in Asian populations). Five polymorphisms have been described, of which only two are associated with enzyme inactivity: C94A, with an activity between 0% and less than 25% of normal and *IVS2* + 21AC, with an activity of 60% of normal[24].

The results from IBD studies are conflicting regarding side effects (pseudoinfluenza syndrome, rash, and pancreatitis) and bone marrow toxicity of azathioprine (Table 2). These studies, in general, have few patients and an even smaller number of patients with the relevant polymorphisms and have been carried out in different ethnic groups. Reliable conclusions, therefore, cannot be drawn. Pseudoinfluenza syndrome causes a large number of patients to abandon the medication. Currently, he importance of the genotype of ITPase in relation to side effects and early treatment suspension is not known.

Prior to the year 2006, it was believed that the conversion of azathioprine to 6-mercaptopurine (6-MP) was not mediated by enzyme action. That year, Eklund demonstrated that it was catalyzed by the glutathione-S-transferases (GSTs)[47]. This author analyzed 14 variants, where GST-A1, GST-A2, and GST-M1 were the three with the highest enzyme activity. All of them are polymorphic. The studies in patients with IBD only allow us to affirm that individuals with low enzyme activity will have low 6-MP levels and, therefore, will not respond to the medication, while patients with ultra-fast activity are at an increased risk for adverse effects due to high 6-MP levels. If this is confirmed, the clinical application would be evident, since the problem could be overcome by directly prescribing 6-MP at the correct dose.

Other enzymes, such as inosine monophosphate dehydrogenase, hypoxanthine phosphoribosyltransferase, *etc*., have been studied, although their application at the present time is unclear.

Likewise, studies have attempted to determine the relationship between the levels of metabolites (6-TG and 6MeMP) and the response to treatment or development of adverse effects. Once again, the results have been contradictory, with some studies describing a relationship between activity and the metabolite levels, while others indicating the opposite. A meta-analysis suggested that 6-TG levels above 260 pmol/8 × 10^8 imply that the patient has a greater probability of disease remission[48]. Blood 6-TG levels above 400 pmol/8 × 10^8 red blood cells increase the risk of bone marrow toxicity[49], and 6-MeMP levels above 5700 pmol/8 × 10^8 red blood cells increase the risk of liver toxicity[50,51]. At present, metabolite determination is not available on a generalized basis in clinical practice, and its use is controversial. However, in patients lacking a clinical response, it may help in deciding medication changes (Figure 2).

Methotrexate

Methotrexate (MTX) is usually used as an alternative to treatment with TPs in CD both for flare-ups and as a maintenance therapy. Its usefulness in UC is more controversial.

The mechanism of action of MTX in IBD has not been clearly established. The drug is a folic acid antagonist and blocks purine and pyrimidine synthesis. Those tissues characterized by greater cellular regeneration (turnover) show more toxic effects. Consequently, the main adverse effects of MTX are bone marrow suppression, mucositis, and gastrointestinal and hepatic alterations. Folic acid supplementation reduces these side effects, although up to 30% of all patients have to suspend the medication.

Most pharmacogenetic studies of MTX have focused on patients with hematological tumors and rheumatoid arthritis (RA). In both of these disease conditions, the dosage and administration route are very different from those used in IBD; the extrapolation of results is therefore not possible.

Herrlinger has carried out the only study to date in patients with IBD. Patients with the 1298C allele of the enzyme MTHFR are more susceptible to adverse

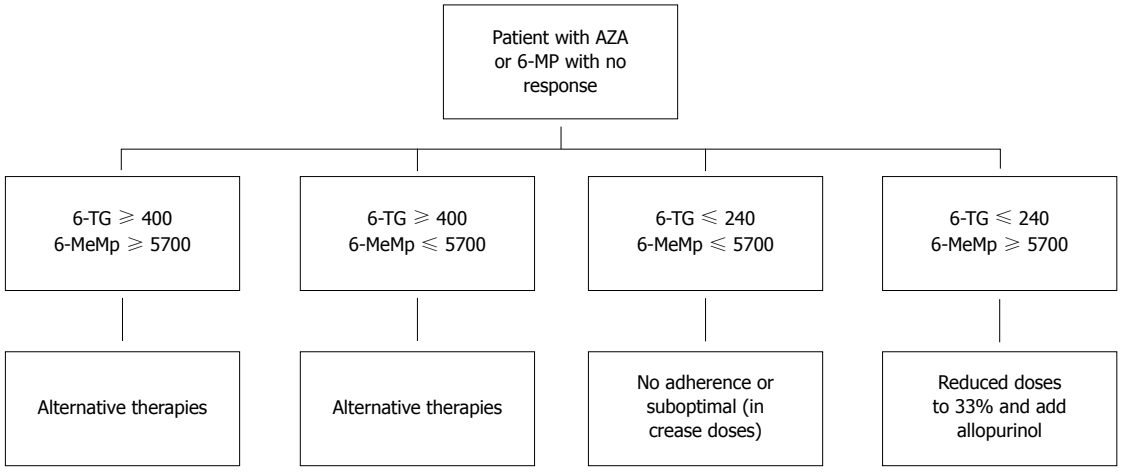

Figure 2 Algorithm of treatment for non responders to thiopurine drugs. AZA: Azathioprine; 6-MP: 6-mercaptopurine; 6-TG: 6-thioguanine; 6-MeMp: 6-methyl mercaptopurine.

effects, although these data are in contrast with those recorded for other diseases[52].

Anti TNF-α drugs

Infliximab (IFX) is a chimeric IgG1 monoclonal antibody (Ab) targeted to TNF-α. It is used for induction and maintenance in patients with moderate to severe flare-ups of IBD. IFX is very useful and has been shown to produce mucosal healing and to reduce the number of flare-ups, hospital admissions, and surgeries. A number of clinical factors indicative of a good response have been described, such as the start of patient treatment at a young age, colonic location of the disease, and associated immunosuppressor therapy. It seems that a shorter duration of the disease, non-smoking, and elevated C-reactive protein levels at the start of therapy also favor a good response[53,54]. Even so, 25% of all patients are primary non-responders, and 20%-30% lose responsiveness over time (secondary non-responders). Most of these latter cases are a consequence of the production of antibodies against IFX[11-13]. Furthermore, the drug is expensive and has potentially serious side effects.

Table 3 summarizes the genetic studies on treatment of IBD with IFX. Studies have been carried out on genes that encode TNF-α, the TNF-α receptor, genes regulating the expression of TNF-α (NOD2/CARD2), apoptotic mechanisms, and other proinflammatory cytokines. To date, it has not been possible to demonstrate an association between drug response and a specific gene. There are two main issues: on the one hand, many studies failed to reach statistically significant results because of the small number of patients involved, and on the other hand, the studies are not always comparable, since different response criteria were used (C-reactive protein, CDAI score, Harvey-Bradshaw index), patients with different degrees of disease activity were included, and different doses were administered.

Perhaps, in the near future, information regarding the genotype of TNF-α and its receptor may help us to identify non-responders to anti-TNF-α therapy.

PHARMACOKINETICS

Understanding the pharmacokinetics of a drug is very important when adjusting the dose required to guarantee therapeutic concentrations, since many factors influence drug concentration in blood. In clinical practice, the determination of blood drug levels has been used to monitor treatments with drug substances that have a narrow therapeutic margin or window. In IBD, such monitoring has been applied to cyclosporine A and tacrolimus; and in recent years, it has become particularly important in the management of anti-TNF-α drugs.

Differences in the administration route, degradation, and clearance of anti-TNF-α determine its concentration in blood and, in turn, its treatment response. In this regard, achieving adequate blood drug levels is correlated with clinical and endoscopic remission of the disease[71,72]. When the anti-TNF-α drug is administered intravenously (*e.g.*, IFX), the maximum blood drug concentration is reached immediately after infusion, with little variability among patients. However, when the anti-TNF-α drug is administered subcutaneously (*e.g.*, antidrug antibodies (ADAs), golimumab, and certolizumab), the maximum concentration is reached after approximately 10 days, and the bioavailability ranges between 50%-100%[73,74].

The clearance of anti-TNF-α drugs from blood is complex and multifactorial. Variables that increase drug clearance include those that depend upon or reflect the severity of the disease (hypoalbuminemia, decreased hemoglobin levels, C-reactive protein elevation, TNF-α, leukocytosis, and increased IFX intestinal losses), demographic parameters (increased body mass, male gender, and age under 40 years) and immunogenicity

Table 3 Genetic association studies of infliximab response in inflammatory bowel disease patients

Study (year)	Patients recruited	Response criteria	Genes investigated	Conclusion
Taylor *et al*[55] (2001)	75	CDAI	Polymorphims TNF/LTA region	Homozygosity for the LTA 1-1-1-1 haplotype may identify subgroups with poorer response
Louis *et al*[56] (2002)	226	CDAI	TNFA	No association with treatment outcome
Mascheretti *et al*[57] (2002)	90 444	CDAI	TNF and TNFR polymorphism	196Arg homozygotes had poorer clinical response than 196Met heterozygotes and homozygotes (P = 0.036) No predictive treatment outcome
Mascheretti *et al*[58] (2002)	534	CDAI	CARD15/NOD2	A strong relation to susceptibility to CD but not association with treatment outcome
Vermeire *et al*[59] (2002)	245	CDAI	CARD15/NOD2	Not predictive of treatment outcome
Pierik *et al*[60] (2004)	166	CDAI	TNF/TNFR	Biological response to infliximab was lower in patients carrying TNFR1-36G
Matsukura *et al*[61] (2008)	80	HBI	TNFRSF1A TNFRSF1B	28% of G allele heterozygotes and homozygotes responded compared to 73% of A allele homozygotes (P = 0.04) 5% of patients with AT haplotype responded compared to 20%-40% of patients with other haplotypes (P = 0.01)
Louis *et al*[62] (2004)	200	CDAI	FcγRIIIa	Positive (V/V genotype) association with good treatment outcome
Urcelay *et al*[63] (2005)	40	CDAI	IBD5(5q31)	Polymorphims TT is associated with negative response
Hlavaty *et al*[64] (2005)	287	CDAI	FASL/CASP9	Positive association
Hlavaty *et al*[65] (2007)	287	CDAI	FASL	Negative association (stadistical model)
Willot *et al*[66] (2006)	189	CRP	CRP	Polymorphims evaluated are not associated with treatment outcome
Dideberg *et al*[67] (2006)	214	CDAI	TNF/LTA region	No association
Dideberg *et al*[68] (2006)	186	CDAI and CRP	ADAM17	Minor allele homozygotes for each SNP associated with clinical response (P < 0.002)
Jürgens *et al*[69] (2010)	90	CAI	IL-23R IL-2/IL-21	Homozygous carriers of IBD risk-increasing IL-23R variants more likely to respond to infliximab than homozygous carriers of IBD risk-decreasing IL-23R variants (P = 0.001)
Dubinsky *et al*[70] (2010)	94	HBI and Partial Mayo score	rs2241880 2q37/*ATG16L1* rs2188962 5q31 rs6908425 6p22/*CDKAL1* rs762421 21q22/*ICOSLG* rs2395185 6p21/*HLA-DAQ1* rs2836878 21q22/*BRWD1*	Six known susceptibility loci associated with primary nonresponse (P < 0.05). Only the 21q22.2/BRWDI loci remained significant in the predictive model

Aailable from: URL: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4419078/.

(the development of antibodies against the drug)[75]. The concomitant use of immunosuppressors (TP drugs and MTX) is associated with decreased immunogenicity and increased anti-TNF-α drug levels.

Up to 40% of all patients that respond to anti-TNF-α drug treatment will require one or more dose adjustments in order to maintain treatment efficacy. After 1 year of treatment, efficacy is maintained in only one-third of all responders[76].

The need for dose adjustment of these drugs may occur at two time points during treatment: at the start (primary failure) or during the maintenance phase (secondary failure).

Primary failure, or a primary lack of response, refers to the absence of improvement in signs and symptoms of the disease that leads to treatment suspension during the induction phase[77]. The time point at which this primary lack of response is assessed varies among different studies. In patients treated with IFX, as in the ACCENT Ⅰ trial[78], assessment was made 2 wk after the first IFX infusion. In contrast, assessment in the ACCENT Ⅱ trial was made 10-14 wk after induction[79]. In studies including patients treated with ADA, the response was assessed in week 4 in the CLASSIC Ⅰ trial[80] and in week 6 in the CLASIC Ⅱ trial[81]. Among the different published studies, the lack of primary response to treatment rate with anti-TNF-α drugs varied from 10%-40% and was found to be higher in UC than in CD. Lack of response was noted particularly in severe presentations of UC. This observation could be explained by increased drug clearance as a result of a greater inflammatory intestinal surface[77].

Secondary failure, or secondary lack of response, is defined as failure occurring in the course of treatment in those patients that have responded to induction therapy and is observed in approximately 13% of the patients treated with IFX[82] and in 10%-24% of those treated with ADA[83]. Failure is more frequent in the first year of therapy.

Table 4 and Table 5 describe the risk factors asso-

Table 4 Risk factors associated to primary failure

Crohn's disease	Ulcerative colitis
Duration of the disease > 2 yr	Old age
Smoking	Anti-neutrophil cytoplasmic (ANC) antibodies
Extensive small bowel involvement	Negative antibodies against *Saccharomyces cerevisiae*
Low C-reactive protein levels	Previous exposure to anti-TNF-α drugs
Genetic mutations or polymorphisms of the apoptosis and caspase 6 genes and locus IBD 5	

IBD: Inflammatory bowel disease.

Table 5 Risk factors associated to secondary failure or loss of response

Individual differences in bioavailability and pharmacokinetics
Symptoms not due to inflammatory bowel disease
Lack of adherence to therapy
Drug loss in stools
Intermittent treatments
Non-inflammatory symptoms
Structuring disease pattern
Smoking
Development of antibodies

ciated with primary and secondary failure[11,84].

In secondary failure, the most widely investigated factor is the development of ADAs. When a loss of treatment response occurs and once the possible existence of intercurrent processes and lack of adherence to therapy have been discarded, the patient requires dose adjustment or a switch to some other molecule. In this context, it is very useful to know the blood drug levels and whether or not ADAs have developed.

The development of ADAs is conditioned by the patient immune condition and is much more common in intermittent treatments (over 60% of the cases) than in maintenance therapy (10%-20%). Furthermore, those patients who have developed ADAs and remain without treatment for extended periods exhibit slow clearance of these antibodies[85].

Many studies have been carried out to determine when and how antibody titers should be measured in order to perform the necessary adjustments and to define the interval during which therapeutic blood drug levels are present. In routine clinical practice, if this is not possible, dose adjustment is made empirically, shortening the interval between doses or switching to another molecule when loss of response occurs or an immune-mediated reaction is observed. Three different methods can be used to measure drug levels.

The most widely used option is the enzyme linked immunosorbent assay (ELISA), which can also be used to determine ADA titers. This technique requires the anti-TNF-α drug to be undetectable in blood, since it binds to the antibodies and forms immune complexes that are not detected.

Radioimmunoassay (RIA) is similar to ELISA, although its use is limited since it involves the use of a radioactive reagent. Furthermore, as for ELISAs, it cannot detect the presence of ADAs if there are detectable drug levels present in blood.

Another method for determining the drug and antibody is variable mobility testing, which allows for the detection of ADAs (against IFX and ADA) in the presence of drug in blood.

The determination of anti-TNF-α drug levels is performed immediately before administration of the next dose. ADAs are only detected when the drug levels are undetectable, since the most widely used technique in clinical practice is the ELISA.

Different studies have correlated the development of ADAs with an increased risk of infusion reactions and increased drug clearance from blood[86]. However, not all studies have established correlations to loss of response. In this regard, a systematic review published by Chaparro *et al*[87] found no differences between maintenance or loss of response according to whether ADAs develop or not, while the meta-analysis conducted by Nanda *et al*[88] found that patients who develop ADAs have a 3-fold greater risk of loss of treatment response than patients who do not develop such antibodies.

Although there is great variability among studies in defining the minimum effective concentration of anti-TNF-α drugs, the determination of drug levels has been correlated with improved disease control and to clinical and endoscopic remission. This is important in designing treatment algorithms[71]. To date, the management approach in clinical practice depends on the drug values and on the presence or absence of ADAs (Table 6).

Current studies are evaluating the role of routine anti-TNF-α drug level measurements in blood during therapy, as is done with other drug substances, in order to facilitate better dose adjustment[86]. However, the different studies propose different cutoff values when defining the therapeutic range. Therefore, studies are needed to determine the adequate therapeutic interval or window.

FUTURE TREATMENTS

The biological drugs currently authorized for the treatment of IBD are monoclonal antibodies targeted to TNF-α (IFX, ADA, certolizumab, and golimumab) and monoclonal antibodies targeted to the leukocyte integrins (natalizumab, approved by the United States Food and Drug Administration (FDA) for refractory CD; and vedolizumab, approved by the European Medicines Agency (EMA) and the FDA).

A number of lines of research have been developed with the aim of blocking the inflammatory process at

Table 6 Treatment algorithm according to antidrug antibodies and drug levels

Anti-TNF-α drug levels	Antibodies	Action
Low	Negative	Increase dose
Low	Positive	Switch drug
High	Not determined	Switch to a drug with a different mechanism of action

different levels. In the coming years, new drugs will be introduced that will contribute to the expansion of therapeutic options.

The drug options that are presently in the most advanced stages of development are described below.

Interleukins

Interleukins (ILs) are soluble inflammatory response messenger (signaling) molecules. Their role in IBD has been clearly established, and research in this field has been particularly wide-ranging and advanced[89].

Anti-IL-12/23 drugs: Ustekinumab is the drug with the most advanced results available to date. Ustekinumab is a fully humanized IgG1κ anti-IL-12/23 monoclonal antibody. It specifically binds to the p40 protein subunit shared by both of the mentioned ILs. Binding prevents the mentioned subunit from interacting with the IL-12Rβ1 receptor protein, which is expressed on the surface of immune cells - thereby inhibiting innate and adaptive immune response stimulation. In an inflammatory environment, naïve CD4+ T cells are induced to interferon (IFN)-γ producing Th1 cells by the action of IL-12 and to Th17 cells by the action of IL-23. The Th17 cells, in turn, are responsible for the production of proinflammatory cytokines, such as IL-17, IL-17F, IL-6, and TNF-α[90,91]. Blocking this pathway has been successfully employed in animal models[92,93], and both ILs play a key role in the inflammatory processes of CD[91,94,95].

The results of a first double-blind and placebo (PB)-controlled phase Ⅱ clinical trial were published in 2008[96]. This study had a complicated design that included two patient populations. In population 1, the results at the primary endpoint were discouraging, with a clinical response rate in week 8 of 49% in the group of patients treated with ustekinumab *vs* 40% in the PB group (P = 0.34). However, in a subgroup of 49 patients previously treated with IFX, statistical significance *vs* PB was reached, with a response rate of 59% and 26%, respectively (P = 0.05), in week 8. In population 2, the clinical response rate with ustekinumab in week 8 was 43% in the subcutaneous treatment group and 54% in the intravenous treatment group. Failure of the study to confirm the primary endpoint was attributed to the high percentage response observed in the PB group. No serious adverse events were detected in week 8, and the recorded problems were similar to those seen in the PB group. Overall, the final conclusion was favorable regarding the capacity of the active drug to elicit a clinical response in the induction phase.

The CERTIFI trial was published in 2012[97]. This was a randomized, double-blind, PB-controlled phase Ⅱa study on the efficacy of ustekinumab in patients with moderate to severe CD refractory to IFX. A total of 526 patients resistant to treatment (50% of the subjects having received at least two anti-TNF-α drugs) were randomized to three intravenous induction treatment arms (1, 3, or 6 mg/kg of ustekinumab) and a PB arm. The 145 patients responding to ustekinumab in week 6 were randomized to subcutaneous maintenance therapy in weeks 8 and 16 with PB *vs* ustekinumab 90 mg. The primary endpoint was the clinical response rate in week 6, where the recorded percentages were 36.6%, 34.1%, and 39.7% (1, 3, or 6 mg/kg of ustekinumab) *vs* 23.5% in the PB arm. Only the 6 mg dose reached statistical significance *vs* PB. In the maintenance phase, 41.7% of the patients treated with ustekinumab showed clinical remission *vs* 27.4% of the patients in the PB arm (P = 0.03), and the clinical response rate was 69.5% *vs* 42.5%, respectively (P < 0.001). The safety profile was found to be similar to that of other biological drugs.

The publication of the results of three phase Ⅲ clinical trials is currently pending. The first of these studies (UNITI-1)[98] is a randomized, double-blind trial designed to evaluate the efficacy and safety of induction with ustekinumab in patients with moderate to severe CD who have failed or are intolerant to anti-TNF-α therapy. The primary endpoint is clinical response in week 6, while the secondary endpoints are remission and clinical response in week 8. A total of 769 patients have been randomized to three arms: (1) intravenous PB; (2) intravenous ustekinumab 130; and (3) intravenous ustekinumab 6 mg/kg as a single dose. The study ended in July 2013. The second study (UNITI-2)[99] has the same design as the first, although the included patients are naïve to biological drugs and show failure or intolerance to immunosuppressors or corticosteroids. This study ended in October 2014 and includes a total of 642 patients. Lastly, the IM-UNITI[100] trial is also a randomized, double-blind, PB-controlled, parallel group multicenter study. This trial was designed to determine efficacy in the maintenance phase of CD and is currently in the recruitment stage. The study plans to include 1310 patients from the two previously mentioned trials, with conclusion in November 2018.

There are two other anti-IL-12/23 molecules: briakinumab (ABT-874), which targets the p40 subunit, and apilimod mesylate, which is a small molecule administered *via* the oral route that inhibits the transcription of IL-12 and IL-23. The initial results with both molecules have not been significant[101,102].

Anti-IL-6 drugs: Interleukin-6 is a proinflammatory cytokine produced by different types of cells. It

participates in a series of processes including T lymphocyte activation and immunoglobulin secretion through the differentiation of B cells into plasma cells[103,104]. Interleukin-6 exerts its action *via* membrane or soluble receptors[105]. In healthy individuals, the IL-6 levels are low and increase in the context of immune processes[103]. This cytokine is increased in CD in the same way as its soluble receptor, and its levels are correlated with the C-reactive protein concentrations[106,107].

Tocilizumab is an IgG1 monoclonal antibody indicated in RA. It binds specifically to the soluble and membrane receptors of IL-6. In one study, 36 patients with active CD were randomly assigned to two treatment arms (intravenous 8 mg/kg every 2 wk or every 4 wk) or PB[108]. The clinical response rate in the group administered tocilizumab every 2 wk was 80% *vs* 31% in the PB arm, although only 20% achieved clinical remission. The drug was well tolerated, although studies in RA have shown neutropenia, altered liver biochemical parameters, and hyperlipidemia. In this regard, dyslipidemia might prove to be a safety problem of the drug long term[109].

Two phase Ⅱ studies involving two monoclonal antibodies (BMS-945429, formerly ALD518, and PF-04236921) targeted to IL-6 are currently ongoing. No results are yet available, since patient recruitment has ended only recently[110,111].

Other anti-interleukin drugs: Interleukin-2 is another molecule that plays a key role in T cell activation and proliferation.

Basiliximab and daclizumab are monoclonal antibodies targeted to CD25, which is the alpha-chain of the IL-2 receptor. Both drugs are used for the prevention of renal graft rejection. Initial studies with basiliximab documented clinical remission in eight out of 10 patients with UC refractory to corticosteroid therapy[112], and a later study recorded a remission rate of up to 65%, without a control group though[113]. In the case of daclizumab, a comparative study *vs* PB failed to demonstrate positive efficacy results[114].

IL-13 is produced by naive T cells and activates natural killer (NK) cells, which in turn synthesize IL-13. IL-13 has been shown to play a key role in the pathogenesis of UC[115,116].

Two phase Ⅱ trials (IMA-648 [arunkinzumab] and CAT-345 [tralokinumab]) published in 2014 randomized the patients to the antibody[117,118] at different doses (in the case of the first study) or PB. Neither study recorded differences in terms of treatment response or clinical remission. Both studies documented a tendency towards lower activity index scores, and the safety profile was favorable. A third phase Ⅱ study in patients with perianal CD has been designed to evaluate the efficacy and safety of another antibody targeted to IL-13 (QAX576) *vs* IFX for comparison[119]. Ten patients have been included, and results from the trial are pending after the end of the recruitment phase.

Vidofludimus is an immunosuppressor that inhibits the release of IL-17 and IFN-γ [by interfering with the janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway and nuclear factor kappa B (NF-κB)], which blocks the enzyme dihydroorotate dehydrogenase (DHODH). This is a small molecule administered *via* the oral route. The results of the ENTRANCE study, a non-controlled multicenter trial, were published in 2013[120]. This study evaluated 34 patients with corticosteroid-dependent IBD (CD and UC) treated with vidofludimus 35 mg/d orally administered (per os, *po*) over 12 wk. The primary endpoint was clinical remission without corticosteroids in week 12, and this was reached by 56% of the patients with CD and 50% of those with UC. The drug was well tolerated, and no serious adverse events were reported - although additional studies with larger patient series are needed to confirm this finding.

Other targets currently under investigation are antibodies against IL-17 (AMG 827), although the study that has been closed because of worsening of patient symptoms[121], IL-18 (GSK1070806)[122], and IL-21(PF-05230900)[123]. These interleukins had been implicated previously in the pathogenesis of IBD[124-126].

Anti-inflammatory interleukins: Lastly, studies have been performed to evaluate the efficacy of the anti-inflammatory interleukins IL-10, IL-11, and IFN-β. The results to date have not been encouraging[127-131].

Chemokine antagonists

Chemokines are small cytokines that induce chemotaxis by interacting with the chemokine transmembrane receptors bound to protein G[132,133]. Their ligand is CCL25, which in the intestine is fundamentally expressed by the luminal epithelial cells[134]. In IBD, and especially in CD, high levels of CCL25 and of T lymphocytes expressing CCR9 have been detected[135].

Vercirnon: CCX-282B (vercirnon) is a molecule administered *via* the oral route that acts as a CCR9 receptor antagonist and has been suggested to reduce lymphocyte trafficking towards the intestine[136]. The data derived from the PROTECT-1 multicenter trial were published in 2013[137]. This was a double-blind multicenter study randomizing 436 patients with CD to three vercirnon treatment groups (250 mg/d *po*, 250 mg twice daily *po*, 500 mg/d *po*) *vs* PB over an induction phase lasting 12 wk. Subsequently, in the case of clinical response, the patients were again randomized to vercirnon (250 mg twice daily *po*) *vs* PB until week 36. The results were not statistically significant, although in week 8 a tendency towards greater efficacy, in terms of clinical response, was observed in the group administered 500 mg/d *vs* PB

(49% *vs* 60% with odds ratio (OR) = 1.53, *P* = 0.111). In week 52, significance was reached in terms of the percentage of patients in remission (47% *vs* 31% with OR = 2.01, *P* = 0.12). The safety profile was found to be favorable, with similar serious and non-serious adverse events rates in both groups.

BMS-936557: Regarding UC, a parallel line of research is based on IFN-γ-induced protein 10 (IP-10, also known as CXCL10). This protein is secreted by monocytes, endothelial cells, and fibroblasts in response to stimulation by IFN-γ[138]. This protein is implicated in chemotaxis and interaction phenomena with T cells through the CXCR3 receptor[139]. Inhibition of this pathway could incline the Th1 response towards a Th2 response[140]. In patients with UC, CXCL10 is over-expressed in plasma and colon tissue[141], and in animal studies it has shown a reasonable efficacy profile warranting the start of evaluations in humans[142]. BMS-936557 (MDX-1100) is a monoclonal antibody targeted to protein IP-10. Its safety profile has been evaluated in phase Ⅰ studies[143], and based on the evidence obtained in patients with RA[144], a specific double-blind multicenter trial on active UC has been started[145]. This phase Ⅱ trial randomized 109 patients to PB or four doses of intravenous 10 mg/kg BMS-936557 every 2 wk. The primary endpoint (clinical response on day 57) was better among the patients that had received the antibody, although statistical significance was not reached (52.7% *vs* 35.2%, *P* = 0.083). Nevertheless, the results were statistically significant in the group of subjects with the highest titers in blood (87.5% *vs* 37%, *P* < 0.001). The number of infections was greater in the active treatment group (12.7%) compared with PB (5.8%), and drug suspension because of adverse events was necessary in 3.6% of cases. At present, we are awaiting the publication of the results of two other phase Ⅱ trials randomizing patients to different doses of the molecule *vs* PB in application to moderate or severe CD[146] or UC[147].

Other chemokine antagonists: There are other lines of research involving chemokines in autoimmune diseases, such as the binding of CXCL10 to its CXC3 receptor[148], which has been shown to be reduced in patients with CD, in parallel to reduction in the C-reactive protein levels[149]. On the other hand, it has been reported that patients with UC show serum and tissues elevations of exotaxin-1, a chemokine that acts by recruiting eosinophils at the intestinal level.

Bertilizumab is a humanized IgG4 monoclonal antibody that blocks exotaxin-1 activity[150]. A phase Ⅱ study involving 42 patients with moderate or severe UC is planned, with the aim of assessing the efficacy and safety of the drug[151].

Endogenous anti-TNF: TNF-kinoid

Another line of investigation involves the generation of anti-TNF-α polyclonal antibodies through active immunotherapy. This strategy is based on the use of a TNF-α derivative as a vaccine. The compound is known as TNK-kinoid (TNF-K), and while biologically inactive, it can interrupt B cell tolerance of their own cytokines, resulting in the production of high titers of antibodies[152]. A first phase Ⅰ/Ⅱ study was presented at Digestive Disease Week in 2011[153], involving 21 patients with moderate to severe CD assigned to different doses on an open-label basis. The safety profile was found to be favorable, with no serious adverse events, and antibodies were generated in 81% of the cases. Clinical remission in week 12 was achieved by 50% of the patients. The results of a second randomized phase Ⅱ study were published in 2012, involving patients with moderate to severe CD and loss of response or with intolerance to conventional anti-TNF-α drug therapy. The trial included 68 patients randomized *vs* PB into two cross-over treatment arms with intramuscular doses of 180 μg on days 0, 7, 28, 84, 91, and 112, with switching of the active drug to PB in the fifth dose and of PB to the active drug in the third dose in the control group. The safety data were again favorable in this case, with only one serious adverse event related to worsening of CD, although the efficacy results were not reported[154].

Janus kinase antagonists

The Janus kinases (JAKs) are a group of proteins corresponding to enzymes associated to cytokine receptors. They form part of a complex system of signal transmission from outside the cell towards the nucleus, activating transcription of the genes that intervene in important cell processes, such as growth, differentiation, proliferation, or migration. The process begins when the membrane receptor is stimulated by a chemical messenger, *e.g.*, a cytokine. This receptor activates JAK, which undergoes auto-phosphorylation and, in turn, phosphorylates the STAT protein. The latter protein then binds to another phosphorylated STAT protein (*i.e.*, it undergoes dimerization) and is translocated to the cell nucleus where DNA transcription factors are activated. This system, known as the JAK/STAT system[155], has been implicated in the pathogenesis of different diseases[156,157], including specifically IBD[158].

Tofacitinib: This is a small molecule administered *via* the oral route that selectively inhibits JAK1 and JAK3, affecting the signaling pathways of cytokines such as IL-2, 4, 7, 9, 15, and 21[159,160]. Blocking these pathways could suppress the activation and proliferation of lymphocytes while maintaining T cell regulatory function[161,162]. In 2002, the United States FDA approved the drug for the treatment of RA. In the case of IBD, the initial data are not encouraging in reference to CD regarding disease response or remission - although the drug is associated to a significant decrease in biomarkers (C-reactive protein

and calprotectin)[162]. In reference to UC, the results of a randomized, double-blind phase Ⅱ trial involving four treatment doses (0.5, 3, 10, or 15 mg/d *po*) or PB in 194 patients with moderate to severe UC were published[163]. The primary endpoint was clinical response in week 8. In the high dose groups (10 and 15 mg, respectively), the clinical response was greater than in the PB series - statistical significance being reached with the 15 mg dose (61% and 78% *vs* 42%, $P = 0.10$ and $P < 0.001$). Clinical remission reached statistical significance *vs* PB with both doses (48% and 41% *vs* 10%, $P < 0.001$). A subsequent sub-analysis demonstrated improvement in patient quality of life[164]. Further studies involving larger patient samples are needed to confirm the efficacy and safety of the drug.

Inhibition of IL-13: In this line of research, studies have been performed on the inhibition of the mentioned pathway with monoclonal antibodies targeted to IL-13, which is responsible for activating the JAK/STAT pathway[165]. The studies are cited in the section on interleukin antagonist drugs.

Anti-adhesion molecules

Adhesion molecules constitute one of the most advanced lines of research in IBD. Adhesion molecules are transmembrane receptors with three domains (intracellular, transmembrane, and extracellular) that induce cellular changes following stimulation by external molecules. These molecules include the integrins and lymphocyte homing receptors.

Natalizumab: The first anti-adhesion drug investigated was natalizumab, and it consists of an IgG4 monoclonal antibody targeted to integrin subunit α4. Natalizumab initially showed favorable results in CD[166,167], although the risk of progressive multifocal leukoencephalopathy (PML) secondary to reactivation of the JC virus has limited its use[168,169]. Natalizumab acts by blocking the interaction of α4β7 with mucosal vascular addressin cell adhesion molecule-1 (MadCAM-1) and the interaction of α4β1 with vascular cell adhesion molecule-1 (VCAM-1), which is critical to lymphocyte trafficking towards the central nervous system - thereby giving rise to the risk of JC virus reactivation[170].

Vedolizumab: Vedolizumab is another IgG1 monoclonal antibody that binds to integrin α4β7, preventing it from binding to its specific intestinal ligand, MadCAM-1. As a result, T lymphocyte migration towards the inflamed intestinal areas is inhibited. In contrast to natalizumab, it does not bind to integrins α4β1 and αEβ7 and does not antagonize the interaction of integrin α4 with VCAM-1. At present, and based on the results of clinical trials[171,172], vedolizumab has been approved by both the FDA and the EMA for the treatment of patients with moderate to severe CD or UC who fail to respond to conventional treatment or therapy with anti-TNF-α drugs.

AMG 181: AMG81 is another humanized IgG2 monoclonal antibody likewise targeted to integrin α4β7[173]. Early-stage studies warrant the safety, pharmacological, and tolerability profile of the drug. AMG 181 is currently being investigated in the context of two randomized, PB-controlled trials in application to both CD[174] and UC[175]. More evidence will be obtained in the coming years.

AJM300: AJM300 is a small molecule administered *via* the oral route that inhibits the α4 receptor. It is known to inhibit the binding of integrin α4β1/α4β7 - expressing cells to VCAM-1/MAdCAM-1 and has efficacy in the prevention of colitis in animal studies[176]. The results of a double-blind multicenter phase Ⅱa trial were published in 2012[177]. The study involved the randomization of 102 patients with active-moderate UC to receive AJM 300 at a dose of 960 mg or PB three times daily for 8 wk. The primary endpoint was the clinical response rate in week 8, which was found to be 62.7% *vs* 25.5% in the AJM300 group and PB group, respectively (OR = 5.35; $P = 0.0002$). The secondary endpoints (clinical remission and mucosal healing in week 8) were also favorable to the study molecule (23.5% *vs* 3.9% with OR = 7.81; $P = 0.0099$ and 58.8% *vs* 29.4% with OR = 4.65; $P = 0.0014$). No serious adverse events (progressive multifocal leukoencephalopathy) were documented over the short term.

Etrolizumab: Etrolizumab is a humanized monoclonal antibody targeted to the B7 subunit present in integrins α4β7 and αEβ7. The results of a double-blind multicenter phase Ⅱ trial were published in 2013[178]. The study involved the randomization of 124 patients with refractory moderate to severe UC to etrolizumab in two treatment arms (subcutaneous 100 mg monthly or subcutaneous 300 mg monthly plus a loading dose of subcutaneous 420 mg between weeks 0 and 2) or PB. The primary endpoint was the clinical remission rate in week 10, with significant results in both active treatment arms *vs* PB (20.5% and 10.3% *vs* 0%, $P = 0.004$ and $P = 0.049$). In the subgroup of patients naïve to anti-TNF-α drug treatment, the differences obtained with the 100 mg dose were even greater (43.8% *vs* 0%, $P = 0.007$). The adverse event rates were similar in all three groups.

MECA-367 and PF-00547,659: The pharmacological properties of two monoclonal antibodies (MECA-367 and PF-00547,659) targeted to MAdCAM, with inhibition of binding of the latter to integrin α4β7, were presented in 2009[179], but subsequent data are only available for PF-00547,659. In 2011, a double-blind study randomized 80 patients with active UC to different single or multiple subcu-

taneous or intravenous doses of PF-00547,659 *vs* PB. Good efficacy *vs* PB was recorded, with endoscopic improvement of the lesions, a good safety and tolerability profile, and no immunogenicity. Results of the TURANDOT trial were published this year[180]. This is a double-blind, PB-controlled multicenter efficacy and safety study in 357 patients with moderate to severe UC randomized to PB or to doses of the antibody (7.5, 22.5, 75, or 225 mg every 4 wk for three doses). The primary endpoint was the remission rate in week 12, and the secondary endpoints were the response rate and mucosal healing rate in week 12. Remission and mucosal healing were significantly greater in the 22.5 mg and 75 mg dose groups *vs* PB, while response was significantly greater for the 22.5 mg and 225 mg groups *vs* PB. The safety profile remained favorable.

Other lines of research

Laquinimod: Laquinimod is a molecule administered *via* the oral route, with great bioavailability and with purported regulatory activity upon antigen-presenting cells (APCs) and T lymphocytes[181,182]. A phase Ⅱ trial randomizing 180 patients to different doses of active treatment *vs* PB reported that increasing dose was inversely proportional to the percentage response or remission[183]. Specifically, the lowest dose (0.5 mg) elicited a clinical response in 55.2% of the patients treated with the active drug *vs* in 31.7% of the patients administered PB, with respective remission rates of 48.3% *vs* 15.9% in week 8.

Masitinib: Gastrointestinal mast cells are usually found beneath the epithelial surfaces and are able to release cytokines, chemokines, prostaglandins, histamine, and heparin. The proliferation of these cells increases at intestinal mucosal and submucosal levels in CD[184,185]. Masitinib is a selective tyrosine kinase inhibitor that targets the c-kit receptor (expressed by mast cells), platelet-derived growth factor receptor-α/β, lymphocyte-specific kinase, Lck/Yes-related protein, fibroblast growth factor receptor 3, and the focal adhesion kinase activation pathway[186]. A phase Ⅱb/Ⅲ phase trial with 450 CD patients is currently underway with this molecule[187].

Visiluzimab, rituximab, and abatacept: Attempts have been made to invert the natural inflammation process in which T cell proliferation *vs* apoptosis is observed. Visiluzimab (a humanized monoclonal antibody against T cell receptor CD3) and rituximab (a chimeric monoclonal antibody targeted to B cell receptor CD20) were evaluated in IBD, where they were shown to have an unfavorable safety profile[188-193]. Abatacept is a recombinant protein that blocks T cell co-stimulation by the antigen-presenting cells (APCs). Its use has been approved for RA, although the results for IBD have been discouraging[194,195].

Morgensen: Immunosuppressive cytokine transforming growth factor (TGF)-β1 is a secreted cytokine with known functions in growth, proliferation, differentiation, and apoptosis. It has been linked to immune regulating functions, depending on the cell upon which it acts and the environment in which it is found. Diminished TGF-β1 activity has been reported in CD. This is due to the binding of an intracellular protein called SMAD 7 to the TGF-β1 receptor[196]. A molecule known as Morgensen (GED301) was first described in 2001. This is an antisense oligonucleotide that hybridizes to the human *SMAD7* messenger RNA (mRNA) and facilitates RNase H-mediated RNA degradation through a classic antisense mechanism. Its release is pH-dependent; accordingly, it is released in the ileum and right colon[196]. Favorable safety results from a phase Ⅰ study in 15 patients with CD were published in 2012[197]. Recently, a phase Ⅱ trial has evaluated 166 patients with active CD assigned to three active drug treatment groups *vs* PB[198]. The clinical remission rates associated with the two highest drug doses were 55% and 65% *vs* 10% for PB (P = 0.001), while the clinical response rates were 58% and 72% *vs* 18% (P = 0.04). The total and serious adverse event rates were similar, with no reported neoplasms.

RPC1063 and GLPG0974: Lastly, the results of two randomized, double-blind, PB controlled trials were presented at the European Crohn's and Colitis Organisation (ECCO) meeting in 2015 on two new molecules: RPC1063 and GLPG0974.

RPC1063 is a molecule administered *via* the oral route with selectivity for sphingosine 1-phosphate (S1P) 1 and 5 receptor modulator. The study included 197 patients with moderate or severe UC that were administered 0.5 mg or 1 mg of the active drug *vs* PB, once daily[199]. The primary endpoint (the proportion of subjects in remission in week 8) reached statistical significance in the highest dose group (16.4% *vs* 6.2%, P = 0.048). The adverse events profiles were comparable between groups, with approximately 31% of patients experiencing a treatment emergent adverse event.

GLPG0974 is a selective free fatty acid receptor antagonist. Binding of the fatty acids to their receptor induces neutrophil activation and migration. The results were assessed in 45 patients with mild to moderate UC treated with GLPG0974 during 4 wk (200 mg/12 h *po vs* PB)[200]. A decrease in calprotectin levels and myeloperoxidase-positive cells was recorded, although there was no difference in terms of response, clinical remission, or mucosal healing. The safety and tolerability profile was favorable.

Stem cells: Different stem cell therapies have been used in CD and UC. Stem cell therapy involves the use of autologous hematopoietic stem cell transplantation

Table 7 Treatment with herbal remedies

Author	*n*	Producto	Comparador	Indicación	Remisión/response herbal *vs* PB or drug (%)
Langmead	44	Aloe vera	Placebo	Induction remission CU	30 *vs* 7
Ben-Arye	23	Triticum aestivum	Placebo	Induction remission CU	91 *vs* 42
Khan	14	Bovine colostrum enema	Placebo	Induction remission CU	
Sandborn	224	HMPL-004	Placebo	Induction remission CU	38/60 *vs* 25/40
Fukunaga	30	Xilei-san suppository	Placebo	Induction remission CU	46 *vs* 0
Zhang	35	XIlei-san enema	Enema dexametasona	Induction remission CU	
Tang	120	HMPL-004	Mesalazina	Induction remission CU	21 *vs* 16
Gupta	30	Boswellia serrata	Sulfasalazina	Induction remission CU	70 *vs* 40
Cheng	153	Jian Pi Ling tablet	Sulfasalazina Placebo	Induction remission CU	53 *vs* 28/19
Wang	106	Kui Jie Quing enemas	Sulfasalazina Prednisolona	Induction remission CU	72 *vs* 9
Cheng	118	Yukui tang ablets	Prednisolonoa Neomicina Vitamina B	Induction remission CU	33 *vs* 17
Fernández Bañares	105	Plantago ovata sedes	Mesalazine	Maintenance remission CU	60 *vs* 65
Hanai	89	Curcumin	Placebo	Maintenance remission CU	95 *vs* 79
Greenfield	43	Oenothera biennis	Evening primrose oil and olive oil	Maintenance remission CU	
Omer	40	Artemisia absinthium	Placebo	Treatment and prevention recurrence EC	65 *vs* 0
Krebs	20	Artemisia absinthium	Placebo	Treatment and prevention recurrence EC	80 *vs* 20
Gerhardt	102	Boswellia serrata extract	Mesalazine	Treatment and prevention recurrence EC	36 *vs* 31
Ren	20	Tripterygium wilfordii	Placebo	Treatment and prevention recurrence EC	
Holtmeier	108	Boswellia serrata extract	Placebo	Treatment and prevention recurrence EC	60 *vs* 55
Tao	45	Tripterygium wilfordii	Mesalazine	Treatment and prevention recurrence EC	68 *vs* 61
Liao	39	Tripterygium wilfordii	Sulphasalazine	Treatment and prevention recurrence EC	94 *vs* 75

Adapted from Ng *et al*[203].

in CD, mesenchymal stem cells administered systemically or locally in perianal fistulas, and other cell treatments where experience is more limited, such as regulatory T cells and dendritic cells. We refer readers to two recent reviews carried out by our group in this field[201,202].

Herbal remedies: Studies have been made that specifically compare treatment with herbal remedies *vs* PB or even conventional therapy, although there are discrepancies in the results due to the lack of homogeneity among the different studies. The findings in terms of safety are favorable, and the predictable costs are lower than in the case of conventional treatment. We recommend a recent systematic review, which affords a more detailed analysis of this subject[203] (Table 7). Among the different herbal remedies employed, special mention must be made of *Andorgraphis paniculata* extract, known as HMPL-004, which has been found to reduce TNF, IL-1β, IFN-γ, and IL-22 in the development of experimental colitis[204].

Fecal transplant: Fecal transplant is a therapeutic alternative in gastrointestinal processes, such as Clostridium difficile-infection, metabolic syndrome, constipation, pouchitis, irritable bowel syndrome, and IBD[205]. In IBD, effectiveness appears to be related to the stability of the colonization of donated bacteria[206]. The experience in CD is limited to six patients in total[207,208]. In UC, there are data available for up to 106 patients[206,208-212]. The study with more patients included 62 cases with UC, finding clinical improvement in 92% and clinical remission in 68%. In the remaining studies, the results have not been as favorable as the aforementioned studies, with clinical remission ranging from 0% to 30% and clinical response from 0% to 70%. The relatively small number of patients evaluated so far does not allow for the establishment of firm conclusions, but it stresses the importance of the microbiota in the pathogenesis of IBD.

CONCLUSION

At present there are a large number of ongoing studies in various stages of research on new molecules for the treatment of IBD. An analysis of mucosal healing is needed in order to evaluate fully the impact of these therapies. In this way, it is expected to change the course of treating IBD. Among the different alternatives, anti-adhesion molecules and interleukin drugs are promising anti-TNF-α treatments.

With developments in the near future in pharmacogenetics, clinical pharmacology, the use of indices that try to classify patients by defining profiles of severity, and new drug molecules, personalized tailoring of treatment strategies will be possible for IBD.

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TOPIC HIGHLIGHT

2015 Advances in Inflammatory Bowel Disease

Arterial structure and function in inflammatory bowel disease

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Abstract

Inflammatory bowel disease (IBD) is the result of a combination of environmental, genetic and immunologic factors that trigger an uncontrolled immune response within the intestine, which results in inflammation among genetically predisposed individuals. Several studies have reported that the prevalence of classic cardiovascular risk factors is lower among subjects with IBD than in the general population, including obesity, dyslipidaemia, diabetes and hypertension. Therefore, given the risk profile of IBD subjects, the expected cardiovascular morbidity and mortality should be lower in these patients than in the general population. However, this is not the case because the standardized mortality ratio is not reduced and the risk of coronary heart disease is increased in patients with IBD. It is reasonable to hypothesize that other factors not considered in the classical stratification of cardiovascular risk may be involved in these subjects. Therefore, IBD may be a useful model with which to evaluate the effects of chronic low-grade inflammation in the development of cardiovascular diseases. Arterial stiffness is both a marker of subclinical target organ damage and a cardiovascular risk factor. In diseases characterized by chronic systemic inflammation, there is evidence that the inflammation affects arterial properties and induces both endothelial dysfunction and arterial stiffening. It has been reported that decreasing inflammation *via* anti tumor necrosis factor alpha therapy decreases arterial stiffness and restores endothelial function in patients with chronic inflammatory disorders. Consistent with these results, several recent studies have been conducted to determine whether arterial properties are altered among patients with IBD. In this review, we discuss the evidence pertaining to arterial structure and function and present the available data regarding arterial stiffness and endothelial function in patients with IBD.

Key words: Arterial stiffness; Ulcerative colitis; Pulse wave velocity; Crohn's disease; Inflammation; Tumour necrosis factor alpha

Core tip: The prevalence of classic cardiovascular risk factors, including obesity, dyslipidaemia, diabetes and hypertension, is lower among patients with inflammatory bowel disease (IBD) than in the general population. However, the risk of coronary heart disease is increased in IBD patients. Chronic inflammation may explain the difference between expected and observed cardiovascular risk. Arterial stiffness, a marker of subclinical target organ damage and a cardiovascular risk factor, is increased in chronic inflammatory disorders. In this review, we discuss the evidence pertaining to arterial structure and function and present the available data regarding arterial stiffness and endothelial function in patients with IBD.

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INTRODUCTION

The idea that "man is as old as his arteries" was postulated by William Osler more than a century ago[1]. This axiom, initially used only in the setting of atherosclerosis, has also been used in the setting of increased arterial stiffness. Many studies, including a recent meta-analysis, have reported that aortic stiffness predicts an individual's risk of developing cardiovascular disease independently of the classic risk factors[2,3]. Moreover, arterial stiffness and endothelial function have been identified as markers of subclinical target organ damage[4]. As target organ damage predicts cardiovascular death independently of the classic cardiovascular risk factors, it has been suggested that identifying organ damage, particularly among individuals at moderate risk for cardiovascular disease (CVD), may be useful[4]. Among these patients, the presence of increased arterial stiffness is sufficient to reclassify their risk of CVD from moderate to high[4].

A relationship between arterial stiffness and several markers of inflammation has been described in healthy subjects and hypertensive individuals[5,6], as well as in patients with chronic inflammatory disorders[7-10], in whom arterial stiffening occurs independently of atherosclerosis and is related to disease duration[8]. Chronic inflammation has also been linked to endothelial dysfunction[7].

Inflammatory bowel disease (IBD) is a chronic inflammatory condition that results from a combination of environmental, genetic and immunologic factors that trigger an uncontrolled immune response within the intestine in genetically predisposed individuals[11]. The dysfunction of the intestinal immune system and cross-reactivity against host epithelial cells have both been implicated as the primary mechanisms by which said inflammation occurs[12]. Therefore, among patients with IBD, it is reasonable that the chronic low-grade inflammation and the acute inflammation that occur during relapses of the disease may affect arterial properties. Several groups have studied both endothelial function and arterial stiffness in subjects with IBD. In this review, we briefly describe the physiology of the arterial system and the available data regarding both arterial stiffness and endothelial function in the setting of IBD.

PHYSIOLOGY OF THE ARTERIAL SYSTEM

The human arterial system is designed to receive pulsatile blood from the left ventricle and distribute it as a steady flow through the peripheral capillaries. Two distinct functions of the arterial tree may be schematized as follows: the ability (1) to deliver blood from the left ventricle (LV) to the capillaries of organs and tissues (conduit function); and (2) to dampen the blood flow and pressure oscillations generated by the heart, ensuring peripheral organ perfusion at both a steady flow rate and pressure (cushioning function)[13].

The efficiency of the conduit function is a consequence of both arterial diameter and the low resistance offered by large arteries to flow (in the supine position, mean blood pressure drops between the ascending aorta and the arteries in the forearm and leg by no more than 2-4 mmHg).

The thoracic aorta and its principal branches are rich in elastic fibres (elastic arteries). In the abdominal aorta and smaller arteries, the numbers of elastic fibres progressively decrease and are replaced by muscular fibres (muscular arteries). The presence of elastic fibres within the walls of the large arteries enables them to dampen blood pressure fluctuations, confining flow pulsations to the larger arteries, particularly the proximal aorta, and storing the stroke volume during systole. Under physiologic conditions, approximately half of the stroke volume is forwarded directly to the peripheral tissues, whereas the remaining 50% of the stroke volume is momentarily stored within the aorta and the large elastic arteries stretching the arterial walls (Figure 1). Approximately 10% of the energy produced by the heart is stored within the arterial wall by increasing the distension of the arteries. During diastole, the energy imbricated within the arterial wall is discharged, and the stored blood is forwarded to the peripheral tissues, ensuring continuous flow and contributing to the maintenance of sufficient diastolic blood pressure.

With ageing, repetitive pulsations (approximately 35 million/year) cause fatigue and fracture the elastin lamellae of the elastic arteries. Ageing is also associated with a number of molecular changes in the

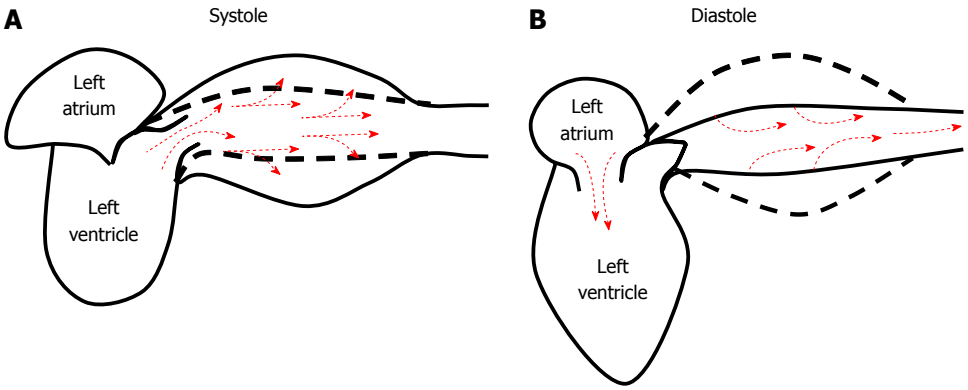

Figure 1 Role of arterial compliance in the damping of blood flow and the pressure oscillations generated by the heart. A: During systole, a portion of the stroke volume is forwarded directly to the peripheral tissues; approximately 50% of the stroke volume is momentarily stored within the aorta and stretches the arterial walls; B: During diastole, the energy imbricated within the arterial wall is discharged, and the stored blood is forwarded into the peripheral tissues, ensuring continuous flow and contributing to the maintenance of sufficient diastolic blood pressure.

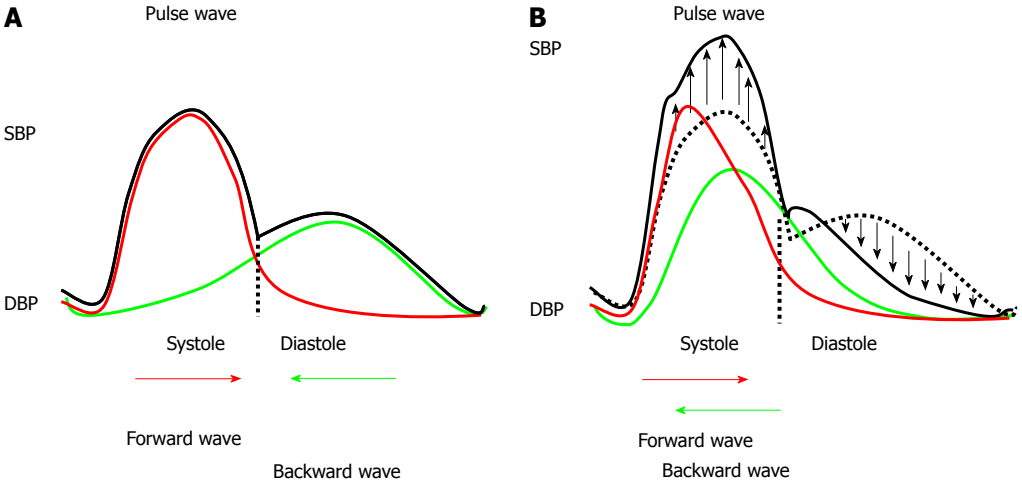

Figure 2 Arterial stiffness and reflection waves. A: Pulse wave in subjects with normal arterial stiffness; B: Pulse waves in subjects with increased arterial stiffness. DBP: Diastolic blood pressure; SBP: Systolic blood pressure.

load-bearing media of the elastic arteries, as follows: the orderly arrangement of elastic fibres and laminae is gradually lost over time, and thinning, splitting, fraying and fragmentation are observed. The degeneration of the elastic fibres is associated with an increase in collagenous material and these changes are often accompanied by calcium deposition and degenerated elastic fibres[14,15]. These processes result in both the stiffening and the dilatation of the large arteries and the early return of the reflected pressure waves to the heart. In the setting of increased arterial stiffness, the aorta and the elastic arteries cannot be stretched during systole. Consequently, the entire stroke volume flows through the arterial system and peripheral tissues only during systole, increasing systolic blood pressure and decreasing diastolic blood pressure.

ARTERIAL STIFFNESS AND REFLECTED WAVES

The integration of the conduit and cushioning functions results in pressure wave propagation and reflection (Figure 2). At each level of the arterial tree, the arterial pulse may be divided into two components, a forward or incident pressure wave, which originates at the level of the left ventricle, and a backward pressure wave, the sum of the reflected waves that originates primarily at the level of the high-resistance arterioles. The forward pressure wave generated within the aorta is propagated to arteries throughout the body. The progressive and physiological increase in arterial stiffness from the proximal aorta to the peripheral muscular arteries, together with the changes in aortic geometry, local arterial branching and luminal narrowing, produces an impedance mismatch and causes partial reflections of the forward pressure waves. The reflected pressure waves travel back to the central aorta and participate in changes in the amplitude of both the systolic blood pressure and the pulse pressure along the arterial tree. The stiffer the elastic and muscular arteries, the faster the forward and backward pulse waves within the arterial tree,

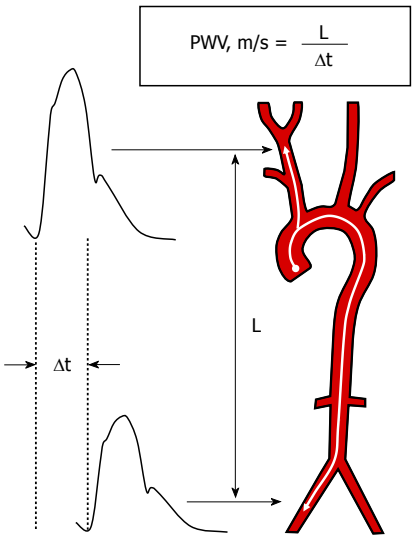

Figure 3 Reference technique utilized to measure carotid-femoral pulse wave velocity. PWV: Pulse wave velocity; L: The distance between the two measurement sites; Δt: The time lag between the pulse waves acquired at the proximal (carotid) and distal (femoral) sites.

and the earlier the return of the backward wave to the ascending aorta. Consequently, at the level of the proximal aorta, the backward wave interacts with the forward wave during diastole in subjects with elastic arteries (*i.e.*, in youth) and during systole in subjects with increased arterial stiffness (*i.e.*, either in the elderly or in the setting of pathological conditions). This causes both a greater peak in aortic pressure during systole and a larger decline during diastole.

HOW TO MEASURE ARTERIAL STIFFNESS IN CLINICAL PRACTICE

Several techniques and devices have been validated to measure arterial stiffness in clinical practice[2]. A direct reflection of arterial stiffness, pulse wave velocity (PWV) represents the gold standard for assessing regional arterial stiffness in daily practice[2]. PWV is the speed at which the pressure wave generated by cardiac ejection is propagated through the arterial tree. PWV is usually measured noninvasively from pressure waveforms obtained transcutaneously at the level of the right common carotid artery and the right femoral artery (carotid-femoral PWV), and the time delay (transit time) measured between the feet of the two waveforms, where the foot of the wave is defined at the end of diastole, when the steep rise of the wavefront begins. The distance covered by the waves is assimilated to the surface distance between the two recording sites[2]. PWV is classically calculated by dividing the distance travelled (in metres) by the time delay (in seconds) between the arrival of the pulse wave at the level of two different measuring sites (Figure 3). Carotid-femoral PWV is equivalent to the stiffness of the aorta. An increased carotid-femoral PWV is considered both a marker of target organ damage and a cardiovascular risk factor[16]. PWV may also be measured at the level of the peripheral muscular arteries (*i.e.*, the brachial artery, carotid-radial PWV); however, the role of muscular artery stiffness in the prediction of cardiovascular risk remains a matter of debate.

PWV should not be confused with blood velocity, as the former is related to the transmission of energy through the arterial wall and varies between 4 and 12 m/s with both age and pressure, whereas the latter is related to the displacement of mass through an incompressible blood column and varies in order of cm/s.

ARTERIAL STIFFNESS AND CARDIOVASCULAR EVENTS, THE PATHOPHYSIOLOGICAL BASIS

In the setting of arterial stiffness, the early return of the reflected waves is primarily responsible for the rise in central systolic blood pressure, the drop in diastolic blood pressure and the associated increase in pulse pressure. The increased central systolic blood pressure is responsible for the augmented systolic work (left ventricular load), left ventricular oxygen requirements and the resultant risk of left ventricular hypertrophy[17]. The physiologic return of the reflected waves at the level of the proximal aorta during diastole is important in maintaining adequate perfusion in the myocardial microvasculature. Therefore, the decrease in central diastolic blood pressure caused by the increased arterial stiffness is responsible for the decreased coronary artery perfusion pressure observed during diastole, as well as the increased risk of myocardial infarction. Moreover, in the setting of left ventricular hypertrophy, the perfusion of the myocardial microvasculature is decreased because the hypertrophied heart contracts and relaxes more slowly, and the duration of systole is subsequently increased, whereas the duration of diastole is decreased. The elevated central pulse pressure that drives cerebral blood flow is responsible for the increased risk of stroke among patients with increased arterial stiffness.

ARTERIAL STIFFNESS AND INFLAMMATION

Several factors have been implicated in the pathophysiology of arterial stiffening. An emerging causal factor is the presence of systemic inflammation. This relationship has been described in various chronic inflammatory disease states, including systemic vasculitis[9], systemic lupus erythematosus[8], rheumatoid arthritis[8] and HIV[18]. Even acute, mild and transient inflammatory stimuli have been associated with the deterioration of the elastic properties of the large arteries[19]. It should

Table 1 Studies that have measured carotid-femoral pulse wave velocity in subjects with inflammatory bowel disease

Ref.	Anti TNF-α therapy (%)	Subjects, *n*			Pulse wave velocity (m/s)		
		IBD		Controls	IBD		Controls
		CD	UC		CD	UC	
Zanoli *et al*[25] (2012)	13	16	16	32	6.5 ± 1.5	6.8 ± 1.3	6.0 ± 0.8[a]
Akdogan *et al*[26] (2013)	5	0	37	30	-	8.9 ± 3.0	7.2 ± 1.7[c]
Theocharidou *et al*[31] (2013)	44	43	23	44	6.8 ± 1.3	6.3 ± 1.1	6.1 ± 0.9
Zanoli *et al*[29] (2014)	19	34	40	80	8.0 ± 1.6	7.8 ± 1.7	7.0 ± 1.1[b,c,d]
Korkmaz *et al*[27] (2014)	2	18	84	74	6.4 ± 1.2	6.6 ± 1.2	5.9 ± 1.2[a]
Theocharidou *et al*[31] (2014)	46	29	15	44	7 ± 1.2	6.3 ± 1.2[d]	6.4 ± 0.9[d]
Aytaç *et al*[28] (2015)	0	25	30	25	9.6 ± 1.4	9.3 ± 1.3	7.6 ± 0.3[e,f]

Data are presented as percentages (%), counts or mean ± SD. [a]$P < 0.05$ *vs* the whole group of subjects with IBD; [b]$P < 0.001$ *vs* the whole group of subjects with IBD; [c]$P < 0.05$ *vs* subjects with ulcerative colitis; [d]$P < 0.001$ *vs* subjects with Crohn's disease; [e]$P < 0.05$ *vs* subjects with ulcerative colitis; [f]$P < 0.001$ *vs* subjects with Crohn's disease. CD: Crohn's disease; UC: Ulcerative colitis; IBD: Inflammatory bowel disease.

be noted that in chronic inflammatory disorders, arterial stiffening may occur independently of atherosclerosis and has been linked to the duration of the inflammatory disease in question[8].

ARTERIAL STRUCTURE AND FUNCTION IN THE SETTING OF IBD

Only a limited number of studies have evaluated endothelial function in subjects with IBD. In a study published in 2007, microvascular dysfunction was linked to the loss of nitric oxide generation by the microvascular endothelium[20]. More recently, endothelial dysfunction was also reported in arterial districts far from the intestinal tract. In 2009 a study described low flow-mediated dilatation and shear stress reactive hyperaemia[21]. These results were confirmed by independent research groups that studied both adult patients[22] and paediatric patients[23]. The number of circulating endothelial precursor cells, markers of endothelial function, was significantly reduced in patients with Crohn's disease (CD), as well as patients with ulcerative colitis (UC), compared with healthy controls, whereas the number of apoptotic endothelial precursor cells was higher in both patients with CD and patients with UC[22]. It was also observed that endothelial function improves following the administration of tumour necrosis factor-alpha (TNF-α) antagonists[24].

Only a limited number of studies have evaluated arterial stiffness in the setting of IBD (Table 1). The first study that measured the PWV in IBD was published by our group in 2012[25]. The stiffness of the elastic arteries (carotid-femoral PWV) and the muscular arteries (carotid-radial PWV) were both increased in subjects with IBD; no significant difference in PWV was noted between the patients with UC and those with CD. These results were subsequently duplicated by independent research groups[26-29]. A correlation between disease duration, a surrogate marker of the chronic inflammatory burden, and arterial stiffness was also reported by our group[25,29] and confirmed by an independent research group[27]. A relationship between arterial stiffness and several markers of inflammation (higher disease activity and more extensive involvement) was also reported[26]. Interestingly, in each of the studies that described increased arterial stiffness in the setting of IBD, only a few subjects (0%-19%) were treated with anti TNF-α therapy. By contrast, in two studies published by the same group[30,31], a higher percentage of patients, approximately 50%, were treated with anti TNF-α therapy; arterial stiffness was found to be only slightly increased in the setting of IBD. These findings were consistent with those of recent reports demonstrating that arterial stiffness[30] and endothelial function[24] both improved following the administration of anti TNF-α therapy among subjects with IBD, findings suggestive of a pivotal role for this cytokine in the pathogenesis of arterial dysfunction.

HOW DOES INFLAMMATION AFFECT ARTERIAL ELASTIC PROPERTIES?

Inflammation may stiffen the large arteries *via* several mechanisms (Figure 4). First, in diseases characterized by chronic inflammation, including IBD, endothelial dysfunction has been reported by multiple groups[7,20-23]. It has been suggested that endothelium-derived factors such as nitric oxide and endothelin-1 may influence the development of arterial stiffness[32,33]. The presence of endothelial dysfunction may result in functional arterial stiffening and the concomitant reduction of nitric oxide bioavailability and the increased activity of an opposing mediator, endothelin-1 (Figure 4A). This mechanism may also be responsible for the reversible increase in the stiffness observed among subjects suffering from acute inflammation.

Endothelial dysfunction may also be associated with both the hyperplasia of vascular smooth muscle cells and the increased synthesis of collagen[34], resulting in *structural* arterial stiffening (Figure 4B). Moreover, the increased levels of circulating inflammatory mediators (*i.e.*, interleukin-1 and TNF-α) promote white blood cell infiltration into blood vessels and changes in

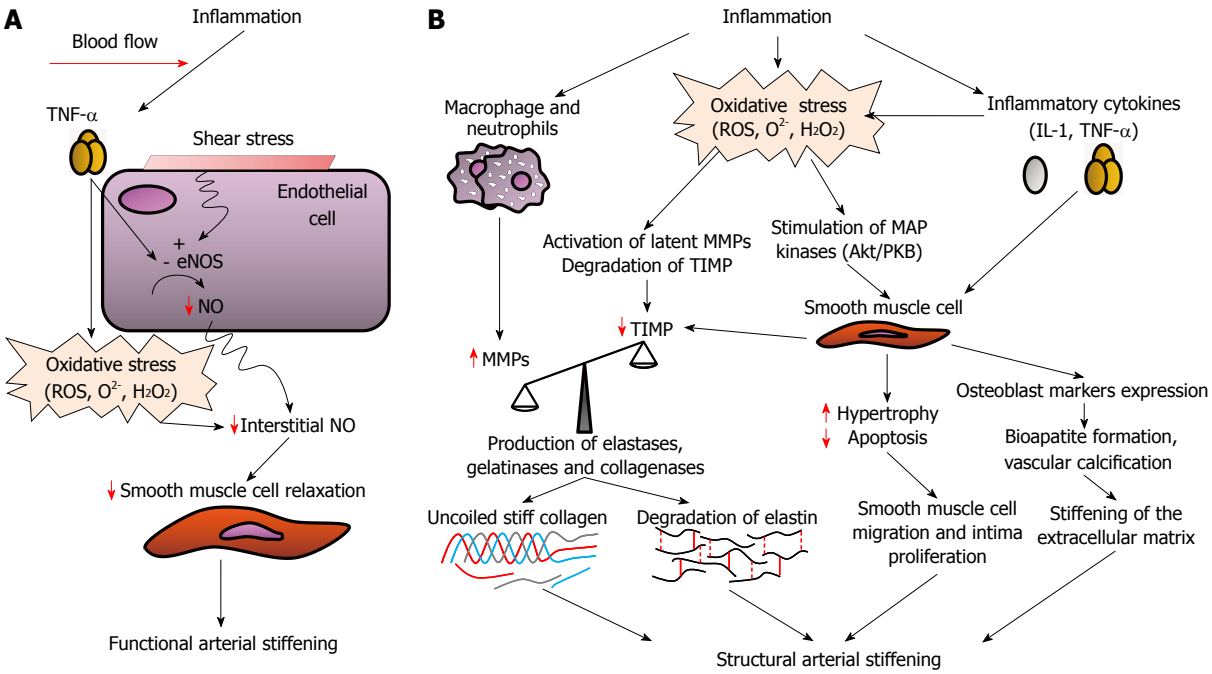

Figure 4 Potential mechanisms by which inflammation can induce functional (A) and structural (B) arterial stiffening. eNOS: Endothelial nitric oxide synthase; H_2O_2: Hydrogen peroxide; IL-1: Interleukin-1; MMPs: Matrix metalloproteinases; NO: Nitric oxide; O^{2-}: Superoxide; ROS: Reactive oxygen species; TIMP: Tissue inhibitor of matrix metalloproteinases; TNF-α: Tumor necrosis factor alpha.

vascular smooth muscle phenotypes, which may release matrix metalloproteinases. The increased fragmentation of elastin molecules may be mediated by the activation of both matrix metalloproteinases and serine proteinases[35,36]. In addition to elastin degradation, matrix metalloproteinases also have collagenolytic activity, which results in the generation of uncoiled and stiffer collagen[35]. The increased matrix metalloproteinase activity may also be mediated by the presence of oxidative stress, which may activate latent matrix metalloproteinases and degrade tissue inhibitors of matrix metalloproteinases, as well as the increased activity of cell adhesion molecules[37,38]. In the setting of chronic inflammation, vascular smooth muscle cells also express osteoblast markers, take up phosphate and produce bioapatite, resulting in medial calcification and reduced vessel elasticity[39], and also produce C-reactive protein (CRP). CRP has an active role in promoting vascular inflammation and reducing endothelial function. Perivascular and vasa vasorum inflammation may result in vessel ischaemia, particularly in the setting of thrombo-occlusion, which may also promote both matrix remodelling and arterial stiffening.

INCREASED CARDIOVASCULAR RISK IN SUBJECTS WITH A LOW PREVALENCE OF CLASSIC CARDIOVASCULAR RISK FACTORS, THE IBD PARADOX

By definition, the higher the prevalence of classic cardiovascular risk factors, the higher the risk of cardiovascular events. However, upon the review of the literature, this axiom does not appear to apply to subjects with IBD. Several studies have reported that the prevalence of classic cardiovascular risk factors is lower among subjects with IBD than in the general population[40-43]. In particular, body mass index and lipid levels are lower in patients with IBD[40-43]. These patients also have lower rates of diabetes, obesity and hypertension[43]. Therefore, given the risk profile of IBD subjects, the expected cardiovascular morbidity and mortality would be expected be lower in these patients than in the general population. However, although the standardized mortality ratio was not reduced[44], the risk of coronary heart disease was reportedly increased in patients with IBD[43,45]. It is reasonable to hypothesize that other factors not considered in the classical stratification of cardiovascular risk may be involved in these subjects. Therefore, IBD may be a useful model with which to evaluate the effects of chronic low-grade inflammation in the development of cardiovascular diseases. In contrast to other clinical models of chronic inflammation in which the prevalence of classic cardiovascular risk factors is comparable with those of the general population, among subjects with IBD, the low cardiovascular risk associated with the low prevalence of classic cardiovascular risk factors may partially offset the cardiovascular burden associated with chronic inflammation[46]. An improved understanding of these concomitant but opposing effects, which are currently not considered in the cardiovascular risk stratification

of patients with IBD, may result in the development of specific programs of intervention aimed at reducing cardiovascular risk. As is the case with other diseases characterized by chronic inflammation[7], increased arterial stiffness may represent a link between chronic low-grade inflammation and increased cardiovascular risk among patients with IBD. Additional studies are necessary to determine whether reduced arterial stiffness and improved endothelial function (with anti TNF-α therapy) decreases the risk of cardiovascular events among subjects with IBD.

In conclusion, there is evidence indicating that in the setting of IBD, as with other chronic inflammatory disorders, endothelial function is reduced, and arterial stiffness is increased. Treatment with anti TNF-α therapy appears to be associated with improvements in both endothelial function and arterial stiffness. Additional studies are necessary to determine whether the improvements in arterial stiffness and endothelial function are associated with a decreased risk of cardiovascular events in subjects with IBD.

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TOPIC HIGHLIGHT

2015 Advances in inflammatory bowel disease

Minimally invasive surgery for paediatric inflammatory bowel disease: Personal experience and literature review

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Abstract

The incidence of paediatric inflammatory bowel disease (PIBD) has dramatically increased in the last 20 years. Although first reported in mid 1970s', diagnostic laparoscopy has started to be routinely adopted in paediatric surgical practice since late 1990s'. Minimally invasive surgery was first limited to diagnostic purposes. After 2002 it was also applied to the radical treatment of PIBD, either Crohn's disease (CD) or Ulcerative colitis. During the last decade minimally invasive approaches to PIBD have gained popularity and have recently became the "gold standard" for the treatment of such invalidating and troublesome chronic diseases. The authors describe and track the historical evolution of minimally invasive surgery for PIBD and address all available opportunities, including most recent advancements such as robotic surgery, single port approaches and minimally invasive treatment of perianal fistulising CD. A systematic review of all series of PIBD treated with minimally invasive approaches published so far is provided in order to determine the incidence and type of patients' complications reported up to present days. The authors also describe their experience with minimally invasive surgery for PIBD and will report the results of 104 laparoscopic procedures performed in a series of 61 patients between January 2006 and December 2014.

Key words: Paediatric inflammatory bowel disease; Ulcerative colitis; Crohn's disease; Laparoscopy; Minimally invasive approach; Complications

Core tip: This review aims at describing the historical evolution of minimally invasive surgery for paediatric inflammatory bowel diseases (PIBD). We will go through all recent technical advancements, provide an overview of our personal experience and perform an extensive systematic review of available data. The series of patients reported so far will be analysed and most relevant issues addressed in details. We do believe that this review will help physicians dealing with PIBDs by reporting and discussing the most advanced surgical opportunities. A special focus on complications and moreover, long-term outcome will help in implementing adequate education for parents.

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INTRODUCTION

Inflammatory bowel disease (IBD) represents a group of chronic relapsing intestinal inflammatory conditions, namely Crohn's disease (CD), ulcerative colitis (UC), and IBD unclassified (IBD-U, a form of colitis whose features made it impossible to discriminate between CD and UC)[1]. Although the exact aetiology of IBD remains unclear, these disorders are thought to result from the interactions of genetics, deranged host immunity and environmental factors[2].

Between 5% and 25% of IBDs occur in children[3]. Although the highest incidence of paediatric IBDs (PIBD) is during adolescence, with two-folds higher prevalence for CD over UC, these disorders can also occur in very young children (< 6 years of age, very early onset IBD). This latter age group is usually characterized by pancolonic inflammation (frequently IBD-U) with severe clinical course and high rate of resistance to immunosuppressive therapy. In these instances a primitive immunodeficiency should always be investigated. At present, the estimated incidence of PIBD ranges between 0.25 and 13.30 per 100000, with a dramatic increase over the last 20 years[2,4].

In children, clinical features of IBDs may be extremely diverse and somehow differ from those of adults, above all in CD. Bloody diarrhoea represents the most common symptoms at onset in UC. Abdominal pain, diarrhoea, weight loss, fever, fatigue, and growth retardation are typically reported in CD with a prevalence ranging between 95% and 25% of cases. As for adults, extraintestinal manifestations are not unlikely in patients with PIBD. Those are reported in 25% to 35% of cases. Interestingly, in children, these symptoms can precede the onset of gastrointestinal disease whereas in adults they tend to occur concurrently with the exacerbation of the disease[5].

Disease localization and severity in children with UC can vary. At onset UC involvement is extensive (pancolitis) in 60%-80% of all patients, while rectosigmoid and left-sided disease are less frequent. Disease extent is consistently associated with disease severity and children have more aggressive disease course with at least one acute severe colitis (ASC) before adulthood[6]. In case of CD, isolated involvement of terminal ileum (± limited to the caecum) is shown in 16% of cases. Isolated colonic disease is reported in 27% and ileocolonic in 53% of cases. Of note, although isolated upper gastrointestinal localization is reported in 4% of patients, 30% have esophagogastroduodenal involvement and 24% jejunal/proximal ileal disease[7]. Perianal disease accounts for 15% of patients. Of note, CD may have insidious onset that leads to delay in diagnosis[6,7].

Medical management of PIBD include nutritional therapy, aminosalicylates, steroids, antibiotics, immunomodulators (*i.e.*, thiopurine, methotrexate), and biologic therapy (infliximab, adalimumab). All drugs can be administered in patients with mild to severe forms of PIBD in order to achieve or maintain remission.

In case of failure of medical treatment, surgical management is indicated to deal with complications. Indications to surgery include bleeding (UC and IBD-U), perforation/abscess (CD), obstruction (CD), stricture (CD), fistula (CD), toxic megacolon (all PIBD), failure of medical therapy (UC), severe growth retardation (UC), and dysplasia or malignancy (all PIBD). In CD a conservative surgical strategy is generally warranted. However, the specific surgical procedure adopted in each case (segmental resection partial colectomy, total colectomy with ileostomy or ileo-rectal anastomosis, and total proctocolectomy with end-ileostomy) depends upon the site of involvement and the type and severity of complications. In UC surgery is curative and includes total colectomy and J-pouch ileo-anal anastomosis with sphincter preservation either resorting to endorectal pull-through or subtotal proctectomy. Ultimately, more than 50% of patients with CD will require resections, whereas 15% to 40% of those with UC will require a colectomy[2,5].

In recent years, as for most of general surgery, surgeons have moved from conventional laparotomy to minimally invasive laparoscopic approach for PIBD. Below the Authors will provide a literature review of recent publications and reports on this regard and will provide details of a series of patients treated at

Giannina Gaslini Institute during the last decade.

METHODOLOGY

Literature review

Two independent investigators performed the literature search, using PubMed, EMBASE, and Ovid database. The search terms were "laparoscopy", "surgery", "children", and "Inflammatory Bowel Disease", or "Ulcerative colitis", "Crohn Disease", and "Indeterminate Colitis". Inclusion criteria were: (1) paper fully written in English language; and (2) patients younger than 18 years of age. All prospective, observational, and retrospective studies were included. Case reports were excluded.

Data were extracted from articles that fulfilled inclusion and exclusion criteria and entered into tables. These data included first Author, country of origin, years of data collection, series size, PIBD types, and incidence of complications.

Personal series

The medical records of all patients affected by PIBD (CD, UC, IBD-U) from January 2006 to December 2014 who underwent minimally invasive surgery (laparoscopic or laparoscopic-assisted) at Giannina Gaslini Children Hospital were reviewed. We recorded demographic data, type of procedure performed (single or staged), operating time, morbidity and length of hospital stay from our centralized operating room database.

Data reporting and statistical analysis

Descriptive statistics were reported as absolute frequencies and percentages for qualitative data, mean ± SD or median and range (based on variability) were used to describe quantitative variables. Differences in the frequencies of each variable were evaluated by the χ^2 test, or by Fisher's exact test, when appropriate. All the statistical tests were two sided and a *P* value lower than 0.05 was considered as statistically significant.

LITERATURE REVIEW

Overall literature review

On the basis of the available literature data, we could address minimally invasive surgery for PIBDs focusing on diagnostic laparoscopy before 2000 and on laparoscopic treatment, afterwards. Furthermore, therapeutic laparoscopy can be divided basing on the adopted surgical procedure[8-26].

Diagnostic laparoscopy

The first report regarding diagnostic laparoscopy dates back to 1975 when Leape *et al*[27] reported the first series of children and infants undergoing diagnostic laparoscopy for various issues, including CD. Diagnostic laparoscopy was used to determine what surgical treatment was eventually required with a conventional laparotomy. Later on, in 1996, Miller and colleagues reported the use of minimally invasive surgery to detect the presence of abnormal mesenteric fat ("creeping fat") in patients with suspected CD. The Authors described 6 children who underwent diagnostic laparoscopy. Three of them were suspected of having CD and underwent resection confirming the diagnosis[28]. Similarly, in 1998, Schier *et al*[29] reported a series of 11 children who underwent diagnostic laparoscopy without major complication. The Authors confirmed the usefulness of direct images in the early stages of CD for a better implementation of adequate medical treatments.

Laparoscopic treatment

Laparoscopy has been adopted in adults since early 90s' either for the treatment of UC or CD with good results[30,31]. In children, therapeutic laparoscopy for IBDs has been introduced since early 2000s'. The first report of laparoscopic treatment of CD dates back to 2002, when Rothemberg reported his preliminary experience with his first 15 segmental bowel resections for CD[10].During the same year, Georgeson reported his preliminary experience with 18 patients with UC who underwent Laparoscopic assisted total colectomy with pouch reconstruction[9]. Both authors reported their preliminary experiences on relatively small pediatric series demonstrating the feasibility of minimally invasive surgery for the treatment of IBDs in children, either CD or UC. Similarly, Proctor in 2002 and Dutta in 2003 reinforced these considerations in two separate reports[8,12]. In particular, Proctor and colleagues reported a comparative retrospective analysis of the results of open *vs* laparoscopic subtotal colectomy for UC, treated in their institution between 1999 and 2001. The authors concluded that laparoscopy requires longer surgery but better cosmetic results with shorter return to normal activities and bowel function, being the incidence of major complications unaffected by the chosen approach. Furthermore, the authors underlined how length of surgery may be improved during the learning curve, as previously reported in adult literature[8].

Up to present years, a number of publications reported extensive use of laparoscopy for the treatment of PIBD and larger series have been reported. So far, the largest series published in international literature is that by Diamond and colleagues who reported a series of 136 patients who underwent 154 laparoscopic procedures for PIBD in the period between 1999 and 2007. The authors reported improved cosmetic results, a reduced hospitalisation but an incidence of major complications comparable to that of open surgery, particularly intestinal obstruction[18] (Table 1).

Surgical procedures

Small bowel and/or ileocolic resection: Either laparoscopically-assisted or total intracorporeal ileo-

Table 1 List of available publications concerning minimally invasive approach for inflammatory bowel diseases in pediatric population (PubMed, EMBASE, Ovid) *n* (%)

Ref.	Country	Year	IBD	CD	UC	IBD-U	Complications	Years
Proctor *et al*[8]	Canada	2002	8	1	5	2	4 (50)	1999-2001
Georgeson[9]	United States	2002	18	0	18	0	NS	NS
Rothenberg *et al*[10]	United States	2002	15	15	0	0	1 (7)	NS
von Allmen *et al*[11]	United States	2003	12	12	0	0	1 (8)	1997-2002
Dutta *et al*[12]	United States	2003	15	15	0	0	2 (13)	1998-2002
Simon *et al*[13]	United States	2003	29	NS	NS	NS	5 (17)	1991-2002
Bonnard *et al*[14]	France	2006	11	11	0	0	2 (18)	1999-2004
Meier *et al*[15]	United States	2007	NS	NS	NS	NS	NS	NS
Fraser *et al*[16]	United States	2010	27	0	27	0	18 (66)	1998-2008
Flores *et al*[17]	Argentina	2010	13	0	13	0	4 (31)	1991-2009
Diamond *et al*[18]	Canada	2010	136	83	50	3	50 (37)	1999-2007
Mattioli *et al*[19]	Italy	2011	16	3	12	1	6 (24)	2006-2010
Laituri *et al*[20]	United States	2011	30	30	0	0	3 (10)	2000-2009
Potter *et al*[21]	United States	2012	9	2	6	1	5 (55)	2010-2011
Linden *et al*[22]	United States	2013	68	0	68	0	13 (19)	2003-2011
Huang *et al*[23]	United States	2013	44	25	16	3	10 (22)	2002-2011
Stephens[24]	Ireland	2013	9	0	9	0	3 (33)	2009-2011
Sharp *et al*[25]	United States	2014	28	28	0	0	8 (29)	2009-2013
Vrecenak *et al*[26]	United States	2014	71	71	0	0	23 (32)	2001-2010
Total			559	296	224	10	158 (29.2)	1991-2013

Case reports or case series have been excluded. This table provides the overall number of IBDs treated in a selected time span. Whenever available, the series were differentiated into patients with UC, CD and IC. Overall, a relatively high prevalence of major complications requiring some sort of surgical intervention has been reported (higher than 29%), still similar or even lower when compared to what previously published for open surgery. Complications rate was calculated basing on series providing incidence (541 overall patients). IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis; IBD-U: Inflammatory bowel disease - unclassified; NS: Not stated.

colic resection and segmental ileal resection have been reported. Those procedures have been adopted in patients with CD and consist of laparoscopy with 3 to 4 ports. The whole bowel is inspected and the site of resection is determined by preoperative imaging matched to intraoperative evidences. The resection-anastomosis can be accomplished *via* a mini-laparotomy (either extending umbilical port incision or by means of a small modified Pfannenstiel incision) through which the bowel is exteriorized, resected and anatomised[13,18]. Alternatively, total intracorporeal resection and anastomosis can be performed as described by Rothemberg and Dutta in their previous reports[10,12].

Although some Authors suggested to resort to the "safer" extracorporeal anastomoses (laparoscopic-assisted approach) due to the inflamed and fragile bowel to be anastomized with staplers[13,14], both alternatives have proved to be safe and effective in experienced hands and are now used worldwide in CD.

Strictureplasty: The Heineke-Mikulicz strictureplasty can be performed with a minimally invasive approach, either totally intracorporeal or laparoscopic-assisted. Although mostly limited to upper gastrointestinal tract or to multisite CD involvement for intestinal preservation, in paediatric settings this procedure can be accomplished with good results. Of note, Romeo and colleagues demonstrated that the recurrence rate of strictureplasty overlaps that of resection-anastomosis, thus making this technique a valid option for selected CD patients at risk for short bowel syndrome following resection[32].

Total or subtotal colectomy: Either in elective or emergency setting, total or subtotal colectomy can be carried out with results that overlap and/or overcome those of conventional open surgery[18]. This procedure has been mostly used for the treatment of UC or IBD-U (occasionally for the treatment of pancolonic CD) and consists of a 4 to 5 ports laparoscopy. The left colon is approached first and divided close to peritoneal reflection with straight or angled stapling devices. Mesentery is divided using available laparoscopic sealing devices (Ligasure® in our experience) and colectomy is carried on in anticlockwise direction. Particular care must be taken when dividing midcolonic vessels in order to avoid lesions of first jejunal loop at Treitz ligament. Similarly, another key-point of colectomy is the right hepatic flexure when surgeons must spare and protect the duodenum and hepatic hilum. Once colonic isolation and resection reaches the caecum, the whole colon can be extracted through the right iliac fossa port and the same wound can be used to fashion the ileostomy. Alternatively, the colon can be extracted by everting the rectum through the anus and stapling the rectum from outside. This alternative approach can turn useful in the elective setting in case of small children, particularly those with IBD-U.

Straight or J-pouch reconstruction? Although a debate regarding the indication to fashion a pouch

during reconstruction after colectomy in PIBD exists, most surgeons resort to this technique. A straight ileo-anal or ileo-rectal anastomosis is used only by a limited number of Authors. In 2006, Tilney and colleagues performed a meta-analysis demonstrating that, though basing on a very few good-quality studies, pouch procedures should be preferred in order to achieve better survival and functional outcome[33]. On the basis of these considerations, although pouch implies frequent endoscopic follow up and a relatively high incidence of pouchitis, (reported in up to 50% of patients[34-36]), pouch procedures represent at the moment the gold standard for reconstruction after colectomy in children with PIBD.

J-pouch ileo-rectal or ileo-anal anastomosis? This is one of the most controversial topics in the surgical treatment of PIBD and represents a key element in the radical treatment of UC/IBD-U and of pancolonic CD. Some surgeons opt for a mucosectomy, endorectal pull-through and J-pouch ileo-anal anastomosis[9]. Others do prefer subtotal proctocolectomy and J-pouch ileo-rectal anastomosis 2-3 cm above the dentate line. The most relevant issues leading surgeons' choice are: (1) relatively high risk of leaving small islands of rectal diseased mucosa in the rectal cuff, in case of endorectal dissection; and (2) relatively high risk of dysplasia and cancer in the 2-3 residual centimetres of rectum left in place in case of J-pouch ileo-rectal anastomosis[9,37]. Regardless of the choice for reconstruction, both options are amenable of laparoscopic approach. The mesentery of the terminal ileum is widened and lengthened and a J-pouch is created through the site of the ileostomy. J-pouch length varies between short pouches[38] and longer ones[18] according to surgeons. The pouch is then returned into the abdomen and the rectal pouch is dissected with a sealing device (*i.e.,* Ligasure®) down to the elevator ani and stapled. A circular stapling device is then inserted into the anus and the ileal-pouch is anastomized 2-3 cm above the dentate line. Alternatively, the endorectal dissection can be accomplished starting 1-2 mm above the dentate line in order to perform a classic endorectal pull-through, as described for Hirschsprung's disease[39].

Single or staged procedures: Total colectomy and J-pouch reconstruction can be accomplished as a single stage (Total colectomy with J-pouch reconstruction and no protective ileostomy), two-stage (Total colectomy with ileostomy followed by J-pouch reconstruction without protective ileostomy) or three-stage procedure (Total colectomy with ileostomy followed by J-pouch reconstruction and protective ileostomy) depending on surgeons' attitude and on patients' general conditions. Protective ileostomy is chosen by most surgeons[8,9,18,38]. Nonetheless, as complications have been frequently related to the ileostomy (*i.e.,* internal hernia, prolapse, adhesion, twisting)[18,19] most surgeons addressed this issue and questioned whether the routine use of protective ileostomy should be abandoned in favour of a strict selection of patients with the highest risk of anastomotic complications (fulminant colitis? very low body mass index? low albumin levels?). Anyway, a three-stage approach is recommended in all emergent situation, *i.e.,* fulminant colitis, patients on high dose steroids, severe malnutrition and IBD-U.

Further technical improvements: Recently, SILS™ devices to perform single incision laparoscopic surgery have been adopted to further contain the trauma of abdominal wall and to improve the outcome of the patients both in terms of reduced pain, shorter postoperative stay, earlier recovery of normal bowel functions and improved cosmetic appearance[25,38]. Finally, robotic surgery is on its way to be applied to J-pouch ileo-rectal or ileo-anal anastomosis, given the possibility to apply this innovative technologies to rectal pouch dissection in order to further minimize the risk of damaging perirectal structures (personal unreported experience).

Complications and long-term functional outcome

Comparing open and laparoscopic surgery for PIBD it comes clear that the incidence of complications does not significantly differ[8,12,35]. Nonetheless, surgery for PIBD is somehow frustrating given the relatively high incidence of surgical problems that can occur in the postoperative period. In fact, complications requiring surgical intervention occurred with an average incidence of 29% (158 out of overall 541 pediatric patients in this review) (Table 1). This percentage is similar or even lower than that reported for open surgery. The most frequent issues are represented by intestinal obstruction, anastomotic leakage or stenosis, pouchitis and faecal incontinence[8,18,19,34,40-44].

In particular, the incidence of complications approaches 55% for UC, being that of intestinal obstruction of around 25% and that of pouchitis of nearly 50%[34]. Similarly, the incidence of complications following surgery for CD can be as high as 33% with both early and late complications reported in pediatric patients[40,41]. This should confirm a higher likelihood of complications for patients with UC.

Functional outcome is acceptable with a wide variability of outcomes in different literature reports. Stavlo *et al*[42] has reported normal continence in 100% of patients in 2003 and Wewer in less than 50% of patients in 2005[43]. This wide range of results is difficult to explain. Though, most surgeons report a relatively high incidence of soiling, urge incontinence and night-time faecal continence issues in the long term[18,19,42-44]. All these issues must be acknowledged to families approaching surgery for PIBD in order to achieve a better education and participation in the long term care of their relatives.

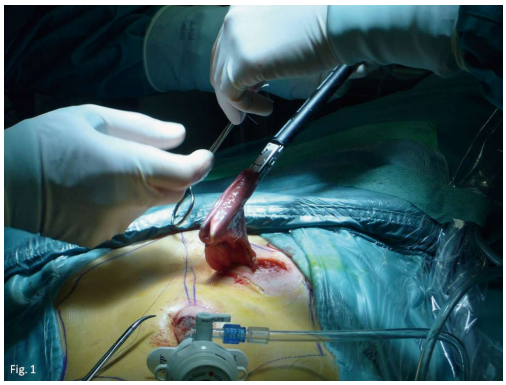

Figure 1 J-pouch is fashioned through the stoma site with a linear stapler.

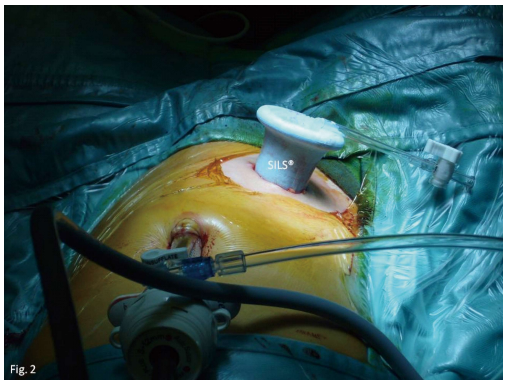

Figure 2 SILS® device can be inserted in the stoma site to help with the perirectal dissection during the J-Pouch Ileo-Rectal restorative anastomoses procedure.

Perianal fistulising CD - minimally invasive surgical options

Since no effective and definitive therapeutic option have been identified for the treatment of perianal disease in children with CD but the need for reduced trauma and sphincters preservation[45], the issue of perianal abscesses and fistula remains difficult to deal with and extremely troublesome both for the patient and the care-giver. In 2013 the World Congress of Gastroenterology implemented shared guidelines and therapeutic options for the medical and surgical management of perianal fistulising CD. The most important aspect is to appropriately select patients for surgery. On this regards, it is widely accepted that surgery for fistulising CD should be used only following complete mucosal healing and no active disease[46]. Those patients with perianal fistulising CD and healthy rectal mucosa are amenable of various surgical options, namely fistulotomy, biological plugs, fibrin glue, advancement flaps, fistula resections (including the so-called "cone-like" resection), stem cells and gracilis muscle interposition[46]. Only some of them can be considered as truly minimally invasive, namely biological plugs, fibrin glue and stem cells based therapy. In particular, the promising fibrin glue treatment showed results similar to those obtained with other established surgical treatment[47]. Although stem cells based therapy proved to be similarly effective, its healing rate of roughly 80% showed a dramatic decrease to nearly 30% over time[46].

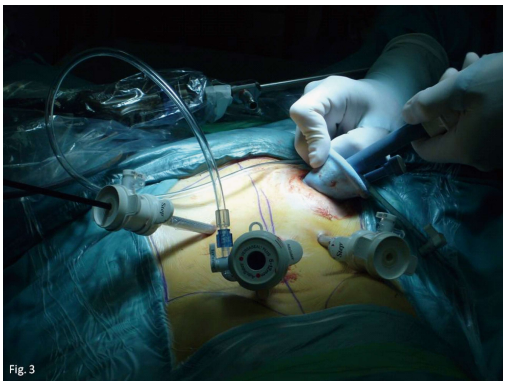

Figure 3 Surgeon can end up with a four-ports procedure using a SILS® device and the ports sites used for the previous laparoscopic subtotal colectomy.

Recently, Meinero *et al*[45] described an innovative and minimally invasive technique for the treatment of anal fistula, named Video Assisted Anal Fistula Treatment. Though based on a small series of patients, Schwandner[48] showed the feasibility of this approach in fistulising CD and the possibility to treat transphincteric, suprashpincteric and rectovaginal fistulae with little to no complications and minimal discomfort. Although results are based on only 11 patients, over 80% success rate and absent continence deterioration are promising aspects for this innovative technique.

PERSONAL SERIES

Between January 2006 and December 2014 (9 years) a total of 104 laparoscopic procedures (98 primary laparoscopy, 6 reoperations for complication) were performed in 61 patients with PIBD. Diagnoses included 39 UC, 20 CD and 2 IBD-U.

Indications to surgery for patients with UC were mostly represented by haemorrhagic colitis, followed by failure of medical treatment and intestinal obstruction/stricture.

Forty-five patients underwent laparoscopic subtotal colectomy (LSTC) and 38 laparoscopic J-Pouch Ileo-Rectal restorative Anastomoses (JPIRA) (Figures 1-3 illustrate our JPIRA technique according to what previously published[38]). In 41 patients LSTC was associated to a protective temporary ileostomy. Four patients underwent LSTC along with JPIRA in a single stage procedure. Definitive ileostomy closure was accomplished in 38 patients.

Fifteen laparoscopic segmental resections have been performed in patients with CD. Two patients required conversion to laparotomy due to the extremely difficult mobilization and manipulation of

Table 2 Personal series of paediatric inflammatory bowel disease treated laparoscopically

	UC	CD	IBD-U	Overall	
Patients	39	20	2	61	
Males				25	M:F ratio 0.7:1
Females				36	
Age at surgery (yr)				9.5 (1.1-20.2)	
Indications					
Haemorrhagic colitis	26	3	2	31	
Failure of treatment	13	3	0	16	
Obstruction/strictures	0	13	0	13	
Other	0	1[1]	0	1	
Procedures					
LSTC	39	4	2	45	Overall 98 procedures
Median operative time (min) (range)				215 (80-350)	
Median length of hospitalization (d) (range)				8.5 (3-11)	
JPIRA	38	0	0	38	
Median operative time (min) (range)				195 (130-260)	
Median length of hospitalization (d) (range)				3.5 (2-11)	
Segmental resection	0	15	0	15	
Median operative time (min) (range)				145 (85-205)	
Median length of hospitalization (d) (range)				7 (2-15)	
Complications (18 events), *n* (%) of procedures	8 (13)	4 (30)	1 (50)	18% of procedures	$P = 0.0806$[2]
Stoma-related issues (6 events), *n* (%) of procedures)	4 (11)	1 (33)	1 (50)	14% of procedures	$P = 0.6322$[3]

[1]Pelvic abscess due to fistulising CD; [2]Statistical analysis was performed to compare the prevalence of complications in CD and UC; [3]Statistical analysis was performed to compare the prevalence of complications related to the stoma (14.3%) and that of complications related to other aspect of surgery (12.2%). Overall, we performed 98 primary laparoscopic procedures and 6 laparoscopic management of surgical complications. Complications were experienced by 21% of our patients following 18% of procedures. Though without statistical significance, complications were more likely to occur in patients with CD when compared to those with UC (30% *vs* 13%). UC: Ulcerative colitis; CD: Crohn disease; IBD-U: Inflammatory bowel disease - unclassified; LSTC: Laparoscopic SubTotal colectomy; JPIRA: J-Pouch Ileo-Rectal restorative anastomosis.

the inflamed and fragile small bowel. Twelve of these procedures were either ileo-colic resections or right hemicolectomies, all with extracorporeal anastomoses except one that was totally intracorporeal performed. Three were segmental small bowel resections with extracorporeal anastomoses. See Table 2 for details.

A total of 18 complications requiring some sort of surgical intervention were experienced by 13 patients (21% of patients being 8 UC, 4 CD and 1 IBD-U). Patients with UC experienced 4 bowel obstruction (all dealt with laparoscopically), 2 anastomotic leaks, 1 endoperitoneal bleeding, 1 anastomotic stricture, 1 ileostomy prolapse, 1 J-pouch prolapse (treated by laparoscopic pouch-pexy). Complications occurred after an average of 2 years postoperatively (range 1 d - 4 years). Patients with CD (20% of patients, 30% of overall procedures) experienced 2 anastomotic leaks, 1 bowel obstruction due to anastomotic stricture, 1 anastomotic leak, 1 pelvic abscess, and 1 ileostomy prolapse. Complications occurred after an average of 52 d postoperatively (range 3-240 d). One patient with IBD-U experienced enterocutaneous fistula at the stoma site.

Long term outcome is being assessed and exhaustive data are now available only for a minority of patients (10 out of 38 who underwent JPIRA, as previously published) with a minimum follow up of 15 mo with satisfactory results in terms of continence, perspectives and cosmetic results[19].

With regard to perianal fistulising CD we routinely apply the so called "cone like resection" with mucosal advancement flaps, which proved to be effective in solving fistulae with promising results. We recently adopted the VAAFT procedure to treat perianal fistula in patients without CD and this minimally invasive approach proved to be feasible and safe in the pediatric population (unpublished data). We now aim at applying this approach to a selected subpopulation of perianal fistulising CD that would benefit of this minimally invasive treatment.

All in all, our experience is in line to what previously published in international literature and confirms the feasibility, safety end effectiveness of minimally invasive surgery for PIBD. Though, complications can still occur and can involve roughly 20% of our patients. Of note, in contrast to what previously published, we observed a higher likelihood of complications in CD (Table 2) but this difference proved not to be statistically significant and deserves a larger series of patients to be confirmed. Anyway, families must be acknowledged on this regard.

CONCLUSION

Advances in surgical treatment of PIBD are striking and include the use of "conventional" laparoscopy, single-incision laparoscopy, robotic surgery and other minimally invasive approaches. Overall technical details and indications do not significantly differ between adults and children. In fact, minimally invasive surgery have been adopted either in the elective or emergency setting thanks to incidence of

complications that proved not to significantly differ from that of conventional open surgery but shorter hospitalization and fewer long term sequelae[49]. According to literature review and personal experience, we can provide good results since indications are based on widely accepted international standards and surgery performed by highly experienced surgeons in third level hospitals. Minimally invasive surgery and fast-track concept of care have been confirmed to fit with PIBD management though a number of problems still occur. In fact, our extensive literature review showed an average incidence of complication of nearly 30% thus confirming the measure of risk for this surgery. On the grounds of these considerations, parents should be adequately acknowledged regarding the risk-benefit ratio of surgery in these high-risk cases. A strict cooperation between surgeons, gastroenterologists, anaesthetists and pathologists is thus required in a multidisciplinary approach to serve the best for our patients.

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TOPIC HIGHLIGHT

2015 Advances in Inflammatory Bowel Disease

Pathological and therapeutic interactions between bacteriophages, microbes and the host in inflammatory bowel disease

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Abstract

The intestinal microbiome is a dynamic system of interactions between the host and its microbes. Under physiological conditions, a fine balance and mutually beneficial relationship is present. Disruption of this balance is a hallmark of inflammatory bowel disease (IBD). Whether an altered microbiome is the consequence or the cause of IBD is currently not fully understood. The pathogenesis of IBD is believed to be a complex interaction between genetic predisposition, the immune system and environmental factors. In the recent years, metagenomic studies of the human microbiome have provided useful data that are helping to assemble the IBD puzzle. In this review, we summarize and discuss current knowledge on the composition of the intestinal microbiota in IBD, host-microbe interactions and therapeutic possibilities using bacteria in IBD. Moreover, an outlook on the possible contribution of bacteriophages in the pathogenesis and therapy of IBD is provided.

Key words: Microbiota; Inflammatory bowel disease; Gut; Bacteriophages; Bacterial therapy

Core tip: Inflammatory bowel diseases (IBD) are chronic disorders of the gastrointestinal tract, with multi-factorial pathogenesis, which affect millions of people worldwide and have a rising incidence. Dysbalanced intestinal microbiota is an important feature of IBD. The relationship between dysbalanced microbiota and IBD is not fully uncovered. We are only beginning to appreciate the role of microbiota in the pathogenesis, progression or prognosis of IBD. In this review, we deal with the composition of gut microbiota, microbe-host interactions, therapeutic potential of bacteria and

discuss the possible roles of bacteriophages in IBD.

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INTRODUCTION

Inflammatory bowel disease (IBD) is a term describing chronic inflammatory diseases of the gastrointestinal tract with a complex etiology caused by various genetic, immunological and environmental factors[1]. IBD refers to ulcerative colitis (UC) and Crohn's disease (CD), which are diseases of the digestive tract with similar clinical, pathological and epidemiological features. They are characterized by recurrent episodes of disease exacerbations with associated abdominal pain, diarrhea, weight loss and rectal bleeding. It estimated that IBD currently affects more than 1 million people in the United States and 2 million people in Europe, with a rising incidence[2,3].

The healthy adult intestine contains about 10^{14} bacteria, a count that is 10x more than the total number of human cells. The total reported number of different bacterial strains in the human microbiota varies with regard to detection method used[4,5]. Recent data have suggested, that the intestinal microbiome comprises approximately 200 strains of bacteria, representing more than 100 bacterial species[6,7]. Advances in metagenomics have uncovered the complexity of this system. More than 90% of these bacterial species fall into three phyla - Firmicutes, Bacteroidetes and Proteobacteria[8].

The gastrointestinal (GI) tract and its microbiome represent a dynamic and mutually beneficial relationship that is thought to be a major determinant of health and disease. The intestinal immune system provides protection to prevent the penetration of an excessive amount of intraluminal bacteria into the systemic circulation. Commensal bacteria activate homeostatic processes based on molecular responses driven by epithelial cells, macrophages, dendritic cells, and T and B lymphocytes that mediate the coexistence with microbes and their products[6]. The gut provides nutrient rich environment for the microbiota, which in turn offers a huge diversity of metabolic functions that include digestion and absorption of non-digestible substrates, a barrier effect against pathogenic microbes and modulation of immune reactions. Disruption of this fine homeostasis on a certain level might lead to the chronic inflammation present in IBD, and also in other chronic inflammatory diseases.

MICROBIOTA AND IBD

Recently, microbial profiles at various stages of colitis have been described and characterized that depend on the time and location within the gastrointestinal tract[9]. It is not yet entirely clear whether changes in the composition of the microbiota are the cause or consequence of inflammatory processes in the intestinal tissue. The most consistent change observed among the vast majority of IBD patients is a decrease in intestinal microbiota diversity, with slightly different findings between CD and UC patients. In CD, a decrease in Firmicutes is often observed, including butyrate-producing bacteria such as *Faecalibacterium prausnitzii*. This leads to the overproduction of pro-inflammatory cytokines and downstream events[10]. In UC, several other groups of bacteria besides butyrate-producing Firmicutes are often reduced, including Bacteroides and Clostridium genera. On the other hand, Enterococcus and Gammaproteobacteria are found in higher amounts in fecal samples from UC patients[11]. However, the presence and abundance of specific bacterial species vary with disease activity and the site of sampling (fecal *vs* biopsy specimens).

Moreover, patterns of gut microbiome dysbiosis in IBD are inconsistent among published studies. A study by Gevers *et al*[12] defined a correlation between a specific microbial pattern and disease status. Samples were collected from multiple locations throughout the GI tract from treatment-naïve pediatric CD patients. The authors concluded that, in the early stages of disease, assessing the rectal-mucosal associated microbiome provides high-value information for a convenient and early diagnosis of CD. In addition, it is known from animal experiments that the presence of specifically altered (procolitic) intestinal microbiota has a direct correlation with the development of colon cancer associated with colitis (colitis-associated cancer - CAC)[13]. Such targeted change in the microbiota (dysbiosis), leading to an increased risk of both, colitis and CAC, is reversible and transmissible to another individual[14]. In this study, dysbiosis-associated disease risk was communicable *via* the gut microbiota to wild-type mice and reciprocal microbiota transplantation reduced disease risk in predisposed mice and led to long-term changes in the gut microbiota composition. Moreover, recent results suggest that intestinal tumorigenesis mediated by bacterial dysbiosis may be communicable through the microbiota among individuals with a genetic predisposition[15]. These studies highlight the potential of preventive and therapeutic manipulation of the intestinal microbiota.

Patients with IBD are at increased risk of developing colorectal cancer and the risk increases with the duration and extent of colitis, positive family history and the degree of inflammation. The pathogenesis of CAC is multifactorial; the key factors are the mucosal

inflammatory response, the presence of oxidative stress and the intestinal microbiota[16]. Some bacterial species of natural microbiota have a protective effect and conversely some contribute to the formation of CAC. The protective function is attributed to probiotic bacteria, which also have a stabilizing effect on the gut flora with the potential to reduce the pro-inflammatory response and thus the risk of development and progression of colitis and related cancer[13]. This effect is even transmissible to wild-type mice, which thus obtained reduced susceptibility to chemically induced colitis[17]. It only remains to be seen whether a dysbiotic state is enough to trigger IBD, which is a major risk factor for CAC. However, it seems that the licensing of dysbiotic microbiota is a key component of disease development. Therefore, manipulation of the gut microbiota certainly brings many opportunities for therapeutic intervention in IBD and CAC.

The whole situation is complicated by the fact that susceptibility to IBD mediated by specific bacterial microbiota also depends on a special diet[18]. This effect is not present in germ-free mice without a natural intestinal microbiota and in mice with a sterilized gut following antibiotic treatment. The absence of an intestinal microbiota thus has a protective effect on the formation and development of colitis and related cancer[13]. In our preliminary experiments, we found that sterilization of the bowel using antibiotics improves subsequent colitis and enhances the therapeutic effect of orally administered bacterial vectors[19]. Although the natural intestinal flora potentiates and promotes chronic inflammation and tumorigenesis, some authors suggest that it can also have the opposite effect - that it limits and reduces chemically induced damage and reduces inflammatory reactions that lead to the development of tumors in the colon[20]. Recolonization of germ-free mice with natural microbiota has been shown to decrease tumorigenesis. As with colitis, a significant change in the composition of the intestinal flora has been described in models of colorectal cancer[21].

The intestinal microbiota is a major component of several physiological processes, including the regulation of body weight and related metabolic balance, the immune system and epithelial cell responses. In recent years, a growing amount of knowledge has been published about the key role of the intestinal microbiota in the pathogenesis of a number of disease conditions, including obesity, cardiovascular diseases, multiple sclerosis, rheumatoid arthritis, diabetes, metabolic syndrome, chronic liver damage and growing evidence suggests chronic lung and kidney disease as well[6,22-25]. Furthermore, it is clear that the presence of a specific intestinal microbiota and interactions between the microbiota and the host organism (its immune system) are crucial for the successful treatment of certain diseases, including cancer, which is not even directly located in the gastrointestinal tract[26]; thus, the gut microbiota affects inflammation and immunity locally at the mucosal level as well as systemically. In this study, antibiotic-treated and germ-free mice showed significantly reduced tumor regression and survival after immunotherapy compared with control mice with natural gut microbiota. This effect was clearly based on decreased bacterial load leading to lower expression of TNF-α in tumors. Moreover, individual bacterial species have been identified that positively (*Alistipes shahii*) or negatively (*Lactobacillus fermentum*) correlate with TNF-α expression in tumors leading to an improved or worsened tumor response to immunotherapy, respectively. This study provided convincing evidence of the crucial influence of commensal bacteria on the therapeutic efficiency in systemic and distant-site diseases and identified individual members of the microbiota that can modulate this effect.

MECHANISMS OF HOST-MICROBE INTERACTIONS IN IBD

There is no question that interactions between microbes and the host play a central role in the development and severity of IBD. A growing body of experimental and clinical evidence has shown that IBD results from a dysregulated immune response to components of the normal gut flora in genetically susceptible individuals. Less is known about the mechanisms of such interactions. However, it is well-known that bacterial exposure is crucial for the development of colitis. In animal studies, genetically engineered mice developed spontaneous colitis when raised under standard conditions, but remained colitis-free when they were housed in germ-free conditions[27]. Moreover, it has been shown that antibiotic pretreatment seems to protect standard mice from the development of chemically induced colitis[19]. Similarly, antibiotic treatment has also been shown to be beneficial in a subset of IBD patients[28].

Lower temporal stability and reduced diversity of the microbiota along with a lower proportion of Gram-positive and a higher proportion of Gram-negative bacteria is frequently reported in IBD patients compared with healthy subjects. In a subset of IBD patients, certain bacterial strains with specific features promote the disease. However, the exact nature of host-microbe interactions that contribute to IBD development has not been assessed for the majority of IBD patients[29]. Apart from experimental studies on animal models of IBD, large genome-wide association studies (GWAS) may provide a relevant clue to explain this complex relationship between host genetic factors and the microbiome. A large meta-analysis was published that described an "IBD genome", *i.e.,* the possible causal genes that point to an essential role for host defense against infection in IBD. These genes are involved in defective processing of intracellular bacteria (NOD2, ATG16L1, IRGM), epithelial barrier

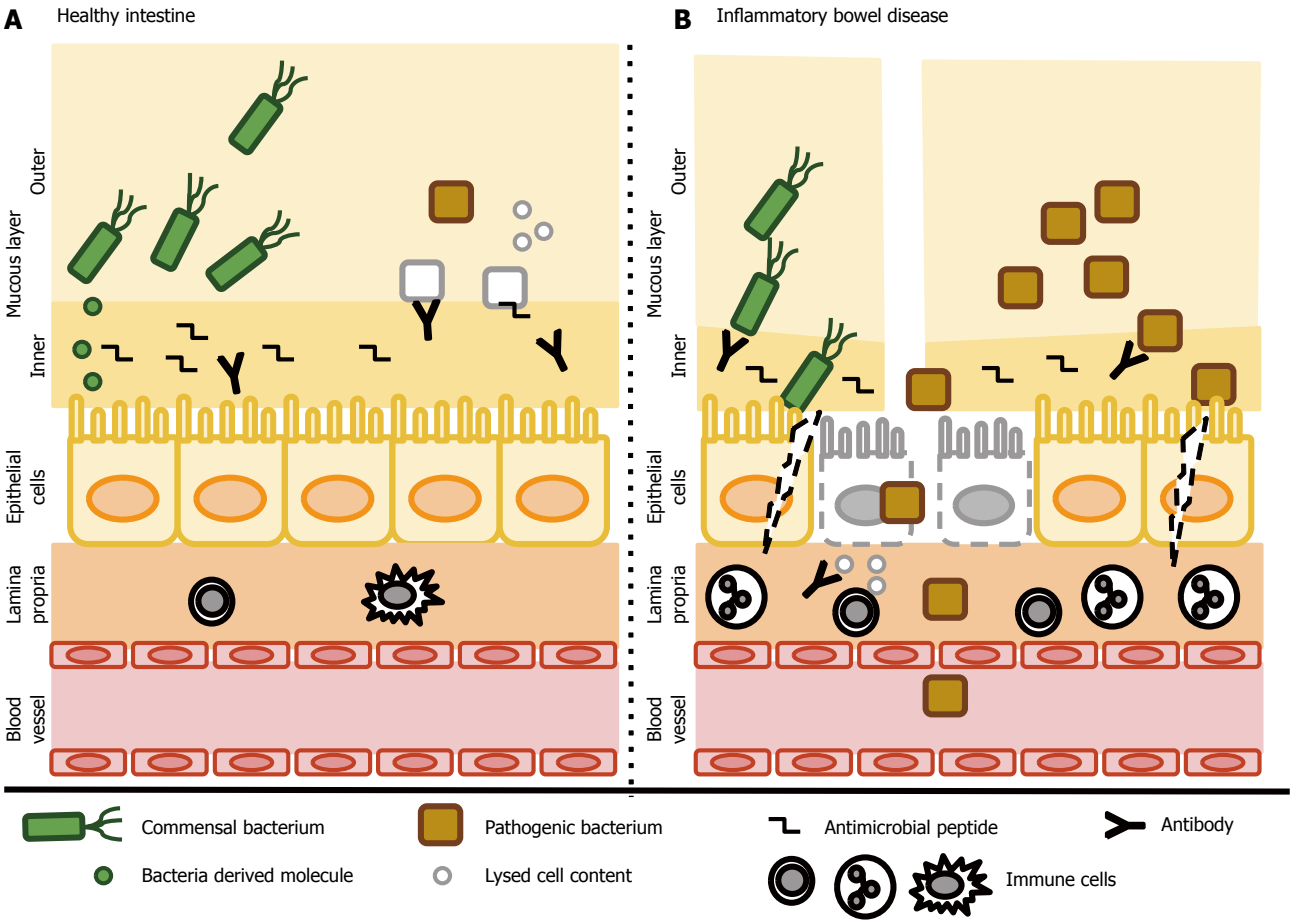

Figure 1 Host-bacteria interactions. A: In the healthy gut, both commensal and pathogenic bacteria reside in the outer layer of the intestinal mucous layer without coming into direct contact with epithelial cells. The inner layer contains abundant antibacterial peptides and secreted antibodies that prevent the invasion of bacteria. Pathogenic bacteria are eradicated by various mechanisms. Commensal bacteria secrete various molecules that help to maintain the intestinal barrier, activate cell survival pathways and suppress inflammatory responses. Epithelial cells form a continuous, selectively permeable layer connected by intercellular junctions. The lamina propria contains only a few resident immune cells; B: In inflammatory bowel disease, the mucous layer is reduced and contains fewer antimicrobial peptides and secretory antibodies. The abundance of commensal bacteria is reduced in favor of pathogenic bacteria and both types enter the inner mucosal barrier and interact directly with epithelial cells. Some epithelial cells undergo cell death and disruption of the epithelial barrier occurs. Cell components are released and trigger further inflammation. Disruption of the epithelial barrier enables bacteria to invade the submucosa and recruit inflammatory cells. Finally, chronic inflammation develops. A dysbalanced immune system leads to the production of antibodies recognizing both commensal bacteria (and further reduce their numbers) and cells of the host, leading to further tissue destruction and inflammation, creating the "circulus vitiosus" typical for inflammatory bowel diseases.

function (HNF4A, CHD1, LAMB1), antigen presentation (HLA-DQA1) and inflammatory mediator production (TNFRSF14, TNFSF9, IL1R2, IL7R). These data confirm the key role of the interaction between the host mucosal immune system and microbes, both at the epithelial cell surface and within the gut lumen. Specifically, the study raises the question of what triggers components of the commensal microbiota to switch from a symbiotic to a pathogenic relationship with the host[30].

There are a number of proposed mechanisms by which the intestinal microbiota interacts with the host cells[31], yet no definite explanation has been generally accepted. It has been found that the molecules secreted from bacteria can enter intestinal cells *via* transporters or endocytosis, and that they activate cell survival pathways. These findings indicate that the interactions between the gut microbiota and host cells are mediated, at least partly, by membrane transport systems[32]. In IBD, membrane function is frequently compromised, thus leading to an altered flow of information from beneficial as well as pathogenic members of gut microbiota. This, along with other processes of IBD pathogenesis, results in the complex clinical appearance of the disease. In general, four mechanisms have been proposed that drive pathogenic immunologic responses to luminal bacteria: (1) bacterial pathogens; (2) dysbiosis of commensal bacteria; (3) host genetic factors; and (4) defective host immunoregulation[33]. A scheme summarizing possible interactions between bacteria and components of the GI tract in health and IBD is depicted in Figure 1.

BACTERIAL THERAPY OF IBD

Although the interaction between the host and intestinal microbiota seems to play essential role in

IBD pathogenesis, standard therapeutic approaches for treating IBD are typically based on suppression of the host immune response; these drugs mainly consist of 5-aminosalicylates, corticosteroids, thiopurines and biologicals[34]. Since a significant amount of IBD patients do not achieve clinical remission after conventional therapy, there is a legitimate need for new therapeutic approaches. Targeting IBD-related microbial dysbiosis can represent an attractive new alternative for IBD therapy. Therapies based on restoration of the intestinal microbiota that have been successfully used in IBD patients include fecal microbiota transplantation, probiotics, prebiotic antibiotics, helminth therapy and dietary polyphenols[8]. The use of antibiotics, probiotics, prebiotics and synbiotics in IBD patients has been extensively discussed in the literature[35]. Such selective manipulation of the intestinal microbiota has been evaluated as an attractive therapeutic option with few adverse effects.

The number of clinical trials that investigated the role of probiotics in IBD remains relatively low. The most extensively tested probiotic preparations include *Escherichia coli* (*E. coli*) *Nissle 1917* and VSL#3 - a highly concentrated mixture of four strains of Lactobacillus (*L. casei, L. plantarum, L. acidophilus and L. delbrueckii subsp. bulgaricus*), three strains of Bifidobacterium (*B. longum, B. breve and B. infantis*) and one strain of Streptococcus (*S. salivarius subsp. thermophilus*). The published results clearly indicate that both the multispecies probiotic VSL#3 and *E. coli* Nissle 1917 can be as efficient as standard pharmacotherapy, but only in UC (both for active disease as well as to induce and maintain remission). On the other hand, results from CD trials are disappointing[33,36,37]. The differential effect of probiotic preparations in UC *vs* CD indicates that IBD is a multifactorial disease with considerable variety in terms of phenotypes and severity.

Probiotic intestinal strains may provide their benefit in various pathological conditions and through different molecular mechanisms. One of these mechanisms which has been recently proposed could be reprogramming of cells in the intestinal wall into the state of pluripotency and subsequent differentiation into a phenotype resistant to pathological factors causing the disease[38]. In our experiments, we showed that dedifferentiation of intestinal cells during the development of colitis may result in resistance of these cells to adverse inflammatory events and ultimately give rise to new and fully functional healthy intestinal tissue. This hypothesis was first formulated theoretically and then supported by the results from a simple experiment[19,34,39].

Novel therapeutic modalities based on the restoration of intestinal homeostasis include fecal microbiota transplantation (FMT), an approach based on the transfer of a stool suspension obtained from a healthy person into the GI tract of diseased patient. FMT restores essential components of the microbiota which could reverse the inflammatory processes observed in IBD. FMT may possibly restore intestinal microbial homeostasis, and preliminary data have shown the clinical efficacy of FMT on refractory IBD or IBD combined with *Clostridium difficile* infection[35,40]. Although the evidence is still limited, the majority of the studies confirmed the efficiency of FMT in the therapy of IBD[37]. Recently, a meta-analysis of clinical studies was performed to evaluate the efficacy of FMT as a treatment for IBD[41]. Overall, 45% (54/119) of IBD patients achieved clinical remission during follow-up. Subgroup analyses demonstrated clinical remission of 22% (95%CI: 10.4%-40.8%) for UC (P = 0.37; I^2 = 0%) and 60.5% (95%CI: 28.4%-85.6%) for CD (P = 0.05; I^2 = 37%). However, more clinical studies have to be performed before FMT can become a part of standard medical care for IBD patients. Randomized controlled trials are currently ongoing that will shed more light into this topic, including an assessment of the long-term consequences of FMT such as infection, cancer, auto-immune and metabolic diseases.

Bacteria as vectors in gene therapy have been known for a long time and have a wide range of action and spectrum of use[42]. Partly justified concerns about the possible pathogenicity slowed their use in the clinic and in the experiment. This problem has been largely overcome by modern genetic engineering. Currently available strains are genetically modified to have reduced and strictly defined virulence, which allows them to enter cells in the target tissue while maintaining safe conditions. Bacterial vectors are especially appropriate for IBD therapy thanks to their natural ability to persist in the intestinal environment. Such bacterial therapy of IBD was first successfully applied more than a decade ago, when the bacterium *Lactococcus lactis* was administered in murine colitis found to secrete interleukin-10 (IL-10)[43]. Similar results were obtained in our experiment where we used recombinant probiotic strains of *E. coli* Nissle 1917 and *L. lactis*, which secreted IL-10 as a treatment for chemically induced colitis[44]. Numerous other studies have confirmed the validity of the bacterial approach in IBD using different combinations of vectors and therapeutic genes[45-50]. Moreover, various bacterial strains have been successfully used for the treatment of cancer[51]. Our results indicate that sterilization of the intestine using antibiotics (the absence of gut microbiota) improves colonization of the gut by administered bacterial vectors and thus enhances the transfer of genes into the intestine using these bacterial vectors[19]. New therapeutic strategies can be expected based on oral administration of genetically engineered live microorganisms producing or delivering anti-inflammatory or other novel agents into the target (intestinal) tissue.

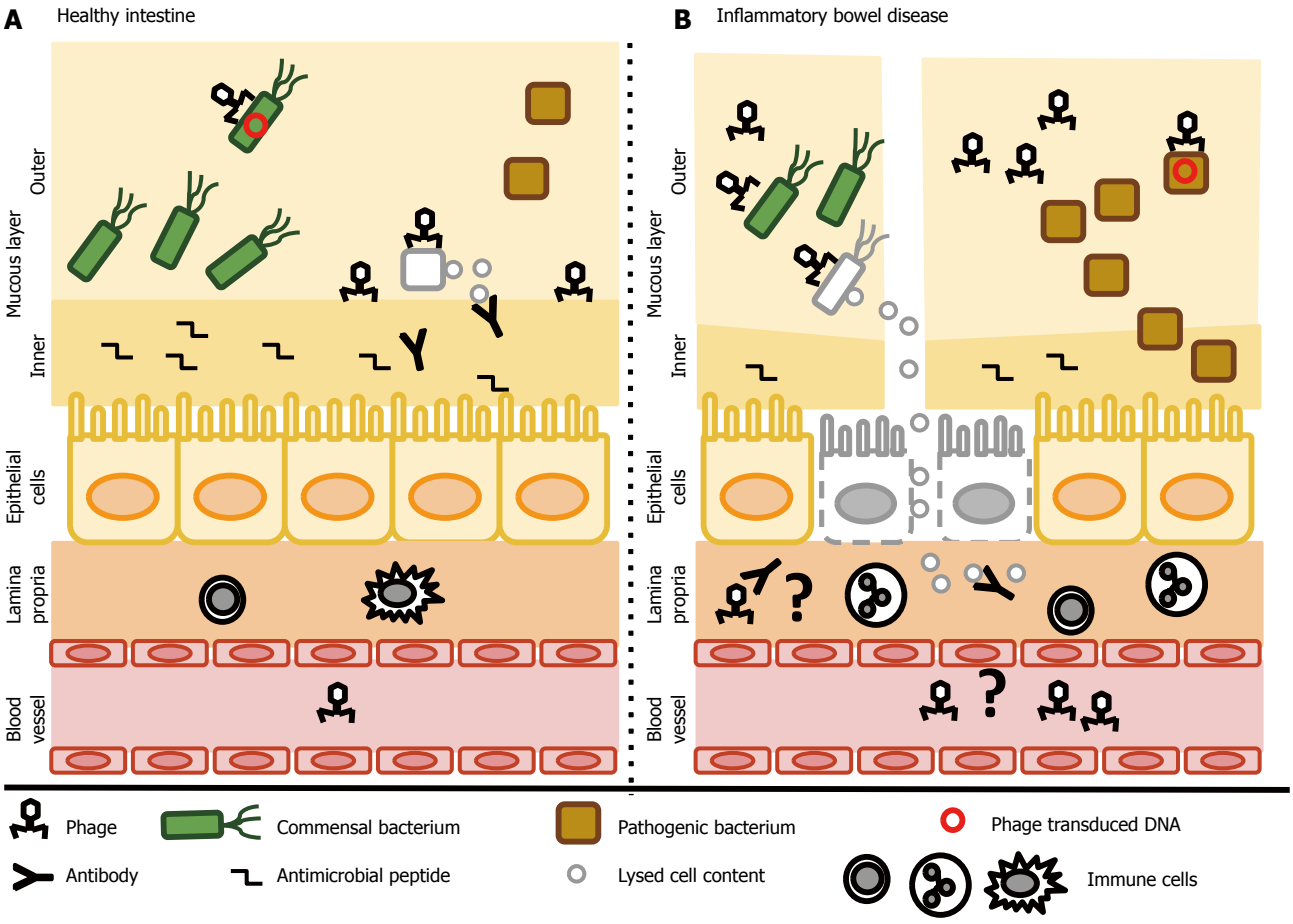

Figure 2 Putative contribution of bacteriophages to regulation of the intestinal bacteria - a simplified scheme. A: In the healthy gut, bacteriophages might increase the fitness of commensal bacteria by the delivery of genes with environmental benefit or contribute to reduction of the pathogenic bacteria. Moreover, phages directly interact with the glycoproteins of the mucous layer and provide protection against invading bacteria. In some healthy individuals, phages have been detected in the circulation, suggesting the possibility that they cross the intestinal epithelial barrier; B: In inflammatory bowel disease, more phages are found in the mucous layer. Higher numbers of phages may be involved in reducing the amount of commensal bacteria, and may drive the transfer of genes with environmental benefit to pathogenic bacteria. Due to the reduced mucosal layer, phage interactions with mucosal glycoproteins may be reduced. Moreover, disruptions in the epithelial barrier might lead to the migration of many phage particles into the lamina propria or even the circulation. In the lamina propria, phages may serve as a local trigger of the immune response. After translocation to the systemic circulation, a systemic immune response might occur.

BACTERIOPHAGES AND IBD

Recent studies have suggested that examination of the gut microbiome should not focus solely on the bacterial composition. Bacteriophages (or phages) are viruses that infect bacteria, but not eukaryotic cells. It is estimated that the human gut contains 10^{15} bacteriophages[52], which accounts for approximately 10^{8}-10^{9} bacteriophages per gram of human feces[53]. The colonization of the gut by bacteriophages increases rapidly after birth, as infant feces contain 10^{8} phage particles per gram of feces at the age of 1 wk[54]. Analysis of the human phage microbiome (phageome) from a fecal sample showed both phages and prophages (phage genomes incorporated in the bacterial genome) being present, while prophages contribute to approximately 28% of all phages[55]. Three dominant families from the order Caudovirales (*Siphoviridae*, *Myoviridae* and *Podoviridae*) have been shown to be the most abundant phages in the intestinal tissue, as confirmed by both electron microscopy in intestinal tissue[56] and by the metagenomic approach in intestinal tissue and gut wash[57]. These three families have been confirmed by other metagenomic analyses, with the addition of the family *Microviridae* in the feces[55]. On the other hand, the metagenomic approach showed that the majority of identified phage sequences are not yet identified (annotated sequences that do not exist in the databases), meaning that more exact information on individual phages in the gut is yet to be discovered[52]. This interest is further supported by the fact that, recently, phages have been shown to be a substantial player in mucosal immunity[58].

Although studies dealing with bacteriophages and IBD are rather scarce, several studies have already described differences in the phage population between CD patients and healthy individuals, both in children and adults[56,57,59]. The diversity of phage genomes was found to be lower in the feces of CD patients than in healthy individuals[59,60]. Interestingly, in the mucosa, CD patients were found to have more detectable bacteriophages than healthy individuals,

but the ulcerated mucosa had a lower phage count than unaffected mucosa[56]. Whether an ulcerated mucosa has more or less bacteria than non-ulcerated parts of the gut is not entirely clear, as some studies suggest no differences between non-ulcerated and ulcerated mucosa[61] and some show less bacteria in ulcerated than in non-ulcerated parts of the mucosa in IBD patients[62]. Fewer bacteriophages could support the model with less bacteria, but this has yet to be determined. This is further complicated by the fact that phages directly interact with mucosal glycoproteins and thus their abundance in the gut is not entirely dependent on the bacteria present[58]. In feces, the relative amount and diversity of bacteria are decreased in IBD[60]. The study also showed an inverse correlation between *Caudovirales* and *Microviridae* in healthy individuals, as well as CD and UC patients. However, significantly higher amounts of *Caudovirales* compared to *Microviridae* were observed only in UC patients.

Given the abundance of phages in the gut, they likely substantially influence the abundance and diversity of bacteria in IBD. The mechanisms by which bacteriophages modulate the actual bacterial flora in the gut are likely multifactorial (Figure 2). Phages substantially contribute to the genetic variability of bacteria by horizontal gene transfer and increasing of the mutation rate[63]. Either way, they substantially influence bacterial fitness[64] and very likely modulate their behavior in IBD. For example, prophages carrying genes encoding antibiotic resistance may act either as procolitic factors, when incorporated into pathogenic bacteria, but may be beneficial when incorporated into probiotic bacteria. Also, stress-induced activation of a prophage dormant in commensal (or pathogenic) bacteria might lead to activation of its lytic cycle and subsequent reduction of the amount of the host bacteria. The vacated environmental niche might be then replaced by pathogenic (or commensal) bacteria with procolitic (or anti-colitic) effects. On the other side, an increase in a certain bacterial strain might lead to increased chance of infection by a specific phage. This is further supported by the fact that CD patients have higher amounts of bacteriophages with less diversity[56], suggesting a regulatory role for bacteriophages in a host-predator manner[55]. On the other hand, the number of bacteriophages may not necessarily correspond to the amount of bacteria[60,65].

Due to the coat proteins of bacteriophages, their effects on IBD could be also immunomodulatory. It has been shown that some phages are able to cross the mucosal barrier and stimulate immunity[66]. Although the available information is rather scarce, phages have been found to modulate both cellular and humoral immunity[66], but the mechanisms are largely unexplored[67]. In IBD, this effect might be further enhanced due to increased permeability of the intestinal mucosal barrier. Several studies have shown the presence of phages ("phagemia") in the bloodstream of healthy individuals[66] and one study has shown phagemia (mycobacteriophages) in patients with CD[68]. This study did not show significant differences in the total amount of phages between healthy subjects and patients with IBD. However, it should be mentioned that the study was performed in the early 1970s and the only detection method available was based on phage titering. Metagenomic approach might provide more information in this subject. Finally, the lytic cycle of phages is followed by lysis of bacterial cells leading to release of macromolecules such as proteins, lipids and nucleic acids which may trigger the immune response and promote inflammation[60].

In recent years, phage therapy has re-gained attention as a therapeutic approach to combat bacterial infections[69]. For IBD patients, this approach could possibly reduce the number of specific disease-associated bacterial strains without a direct negative effect on commensal bacteria. Moreover, metagenomic studies unveiling the "phageome" of the gut of IBD patients might help to develop new strategies or screening methods for the prediction of disease progression, and/or serve as a prediction tool for choosing the optimal therapeutic strategy. On the other side, in light of recent findings focused on phages and their putative role in IBD, especially those that do not depend on bacteria, further studies on safety and efficacy are necessary.

CONCLUSION

The pathogenesis of IBD involves several key processes, including disturbed activation of the mucosal immune system driven by abnormal intestinal microbiota in genetically predisposed individuals. Systematic shifts in the gut microbiome structure and function have been observed in patients with IBD, compared with healthy individuals. However, there are still no definitive microbial pathogens linked to the onset and development of IBD[70]. An overview of the literature has been provided that describes the causes of dysbiosis and the mechanisms evolved by the host to prevent these changes to community structure[71]. Nevertheless, results from previous studies indicate that the taxonomic composition of the microbiome can differ substantially between subjects with the same disease and, thus mere taxonomic characterization might not be sufficient to fully uncover the relationship between the microbiome and IBD. A more relevant and up-to-date method of studying the interactions between microbes and disease seems to be the analysis of microbiome biological properties (functional analysis). Altered intestinal tissue along with microbial dysbiosis both result into significant metabolic shifts within the intestinal microenvironments in IBD[72]. The Integrative Human Microbiome Project (iHMP) is an ongoing multi-omic longitudinal study focused on (1) explaining how the intestinal microbiome may trigger disease activity in IBD; (2) determining if the microbial

composition predicts the risk of exacerbations; and (3) testing whether a successful therapeutic response can be predicted from the stool microbiota[73]. Such an approach should provide a complex view on the dynamics of host-microbiome interactions and link them to specific disease states, including IBD. One of the challenges that have to be addressed is the timing of the administration of bacterial therapeutics. Unlike animal models, most IBD patients are treated after the appearance of serious symptoms, *i.e.,* after the diagnosis of IBD. Therefore, the therapeutic effect might be more variable, insufficient or prone to failure. Nevertheless, ever-growing knowledge about the transmission potential of the healthy gut microbiome supports the rationale of preventive manipulation of the gut microbiota even before the diagnosis and onset of symptoms.

In conclusion, we predict that rigorous gut microbiota profiling will soon become a part of complex phenotypic analysis in IBD patients. Moreover, interventions targeting the microbial composition (including FMT, bacterial gene therapy, synthetic and multimicrobial microbiota substitutes) and the correct timing of these procedures will define the future directions in the field of IBD.

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TOPIC HIGHLIGHT

2015 Advances in Inflammatory Bowel Disease

How should immunomodulators be optimized when used as combination therapy with anti-tumor necrosis factor agents in the management of inflammatory bowel disease?

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Abstract

In the last 15 years the management of inflammatory bowel disease has evolved greatly, largely through the increased use of immunomodulators and, especially, anti-tumor necrosis factor (anti-TNF) biologic agents. Within this time period, confidence in the use of anti-TNFs has increased, whilst, especially in recent years, the efficacy and safety of thiopurines has been questioned. Yet despite recent concerns regarding the risk: benefit profile of thiopurines, combination therapy with an immunomodulator and an anti-TNF has emerged as the recommended treatment strategy for the majority of patients with moderate-severe disease, especially those who are recently diagnosed. Concurrently, therapeutic drug monitoring has emerged as a means of optimizing the dosage of both immunomodulators and anti-TNFs. However the recommended therapeutic target levels for both drug classes were largely derived from studies of monotherapy with either agent, or studies underpowered to analyze outcomes in combination therapy patients. It has been assumed that these target levels are applicable to patients on combination therapy also, however there are few data to support this. Similarly, the timing and duration of treatment with immunomodulators when used in combination therapy remains unknown. Recent attention, including post hoc analyses of the pivotal registration trials, has focused on the optimization of anti-TNF agents, when used as either monotherapy or combination therapy. This review will instead focus on how best to optimize immunomodulators when used in combination therapy, including an evaluation of recent data addressing unanswered questions regarding the optimal timing, dosage and duration of immunomodulator therapy in combination therapy patients.

Key words: Inflammatory bowel disease; Thiopurines; Drug monitoring; Tumor necrosis factor-alpha; Combination therapy

Core tip: Clinicians managing inflammatory bowel disease frequently have to decide whether to use anti-tumor necrosis factor (anti-TNF) therapy alone or in combination with immunomodulators (IM), which requires an assessment of patient factors and the risk/benefit profile of each treatment strategy. Once a decision is made to use combination therapy, questions on how best to optimize IMs must be addressed. Thiopurines, rather than methotrexate, (MTX) are more efficacious and easier to administer, whereas in certain population groups, MTX may be safer. The effective dose of IM may be lower in combination therapy and combination therapy is probably most important in the first 12 mo of treatment. Withdrawing IMs is best done when the patient is in deep remission, ideally supported by the use of therapeutic drug monitoring of anti-TNFs.

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INTRODUCTION

Inflammatory bowel disease (IBD) namely Crohn's disease (CD) and ulcerative colitis (UC), are chronic inflammatory conditions characterized by an exaggerated host immune response to an as yet unidentified antigen, leading to relapsing and remitting inflammation resulting in damage to the gastrointestinal tract. Despite access to an expanding therapeutic armamentarium with the arrival of gut-specific therapies such as vedolizumab and other novel agents targeting key pro-inflammatory cytokines, clinicians still largely rely on the conventional immunomodulators, (IMs) azathioprine, (AZA) mercaptopurine, (MP) and methotrexate, (MTX) and/or anti-tumor necrosis factor (anti-TNF) therapy, (infliximab, (IFX) adalimumab, (ADA) certolizumab pegol and to a lesser extent, golimumab) to treat these diseases. Much has been learnt over the last 15 years of the relative risks and benefits of using these agents, either alone or in combination, however gaps in our knowledge remain as to how IMs are best optimized once a decision has been made to combine them with anti-TNF therapy. This review article begins with a brief outline of the efficacy and safety issues surrounding combination therapy (IM + anti-TNF) and then draws on the available evidence to address some of these unanswered questions (Table 1).

Table 1 Summary and key points

Combination therapy (thiopurines with anti-TNF) is more efficacious than either agent alone in thiopurine-naïve patients with IBD
Combination therapy confers an increased risk of adverse events, of which NMSC, melanoma and lymphoma are the best studied
The benefit of combination therapy is probably due to both an improvement in anti-TNF pharmacokinetics (reduced immunogenicity and improvement in drug levels) and an independent effect of the IM on disease activity
The pharmacokinetic benefits of combination therapy are most important during the first 12 mo of therapy, but may persist beyond this
The optimal dose of IM in this setting may be lower than that used for IM monotherapy, however further studies are needed to confirm this
The risk of relapse after IM withdrawal is highest amongst patients with active disease and positive biomarkers of inflammation or unfavorable anti-TNF pharmacokinetic profiles
Withdrawal of IM should be considered in patients in deep remission after a period of 12 (or perhaps 24 mo) of combination therapy

TNF: Tumor necrosis factor; IBD: Inflammatory bowel disease; IMs: Immunomodulators; NMSC: Non-melanoma skin cancer.

BENEFITS OF COMBINATION THERAPY *VS* ANTI-TNF MONOTHERAPY

The arrival of IFX, and subsequently ADA, both effective therapies for induction and maintenance of remission for luminal and fistulizing CD and UC, revolutionized the management of IBD[1-9]. A common issue faced by clinicians is under what circumstances does combination therapy with an IM offer benefit over anti-TNF monotherapy. Amongst IM naïve patients with moderate-severe CD, the SONIC study (508 treatment naïve CD patients randomized to AZA, IFX or combination therapy) showed that combination therapy was superior to IFX monotherapy with respect to corticosteroid-free clinical remission (56.8% *vs* 44.4%, $P = 0.02$) and mucosal healing (43.9% *vs* 30.1%, $P = 0.06$)[10]. Similar results in moderate-severe UC were seen in the UC-SUCCESS trial, favoring combination therapy (AZA + IFX) over IFX monotherapy for clinical remission, (39.7% *vs* 22.1%, $P = 0.017$) and complete mucosal healing, (29.5% *vs* 11.7%, $P = 0.006$) at week 16[11]. These results should be interpreted with caution as this study was terminated early, and therefore underpowered, and week 16 may be too early for thiopurines to be efficacious; however combination therapy was as effective as, or superior to, IFX monotherapy across a range of secondary endpoints. COMMIT, a 50 wk randomized placebo-controlled trial of CD patients initiated on prednisolone found no benefit of MTX and IFX combination therapy ($n = 63$) over IFX monotherapy ($n = 63$) for the primary endpoint, defined as failure to enter steroid-free clinical remission at week 14, (78% *vs* 76%, P = NS) or failure to maintain remission through week 50, (57% *vs* 56%, P = NS)[12]. When reconciling the opposing findings of combination therapy *vs* anti-TNF monotherapy of SONIC/SUCCESS *vs* COMMIT, several

key differences in study design should be considered. COMMIT used a high dose corticosteroid induction regimen that may have obscured a true benefit of MTX combination therapy over IFX monotherapy. Further, the primary end-point of corticosteroid free remission may have been seen equally between treatment arms due to the enrolment of patients with milder CD activity, a proportion of which may have never failed treatment according to clinical (CDAI) criteria. Of note, in COMMIT, patients randomized to combination therapy had higher median trough drug levels compared to IFX monotherapy (6.35 μg/mL *vs* 3.75 μg/mL, P = 0.08), suggesting a beneficial effect of combination therapy on IFX pharmacokinetics.

Sub-group analyses of RCTs of IFX and ADA for both CD and UC, stratified according to baseline IM use, have failed to show a benefit of combination therapy over anti-TNF monotherapy in achieving clinical remission[1,4,6,7,9,13]. However, a large percentage of patients entered these studies already failing IMs, a key difference from the low proportion of previous IM use in SONIC, SUCCESS and COMMIT. Further, in the ADA RCTs there were high rates of previous IFX failure, (CHARM 49%[6], ULTRA-2 41%[9]) therefore these patients may represent a more treatment-refractory cohort. Data from observational studies has been conflicting with some supporting combination therapy over anti-TNF monotherapy[14-19], whereas others do not[20-23]. Differences in study design; patient populations and endpoints all hamper the strength of conclusions that can be drawn from these studies.

A post-hoc analysis of patient level data, (published in abstract form only) taken from 11 anti-TNF RCTs (IFX, ADA, and certolizumab pegol) found that combination therapy was more efficacious than monotherapy for 6 mo clinical remission in those treated with IFX (OR = 1.79; 95%CI: 1.06-3.01) but not ADA (OR = 0.88; 95%CI: 0.58-1.35) or certolizumab (OR = 0.93; 95%CI: 0.65-1.34)[24]. This may be explained as IFX, a chimeric anti-TNF is more immunogenic than the humanized ADA. A "SONIC-type" study comparing ADA monotherapy to ADA+IM combination therapy is needed before we can say with certainty that combination therapy is more efficacious in this setting.

Taken together the literature suggests that in IM naïve patients with moderate to severe IBD, combination therapy is more efficacious and should be considered over monotherapy with an anti-TNF, and that in IM refractory patients, combination therapy may be important for at least the first 12 mo of anti-TNF treatment.

RISKS OF COMBINATION THERAPY *VS* MONOTHERAPY

Infections and malignancy

Any putative increase in efficacy through the use of combination therapy must be balanced against the risk of adverse events, and infectious complications and malignancy in particular[25]. Randomized controlled trials in IBD have shown no significant increase in infections in patients treated with combination therapy compared with anti-TNF monotherapy. A pooled analysis of 1383 patients, randomized to receive either placebo or IFX, of which 40% received concomitant immunomodulation with AZA, MP or MTX from the landmark ACCENT Ⅰ and ACCENT Ⅱ (luminal and fistulizing CD respectively), and ACT Ⅰ and ACT Ⅱ (UC), studies showed similar rates of both infections (44.1% *vs* 44.5%) and serious infections (3.7% *vs* 3.2%) in those treated with immunomodulator co-therapy *vs* those treated with IFX monotherapy[26]. Similarly, in SONIC serious infections were seen in 4.9% *vs* 3.9%, (P = 0.79) of those treated with IFX monotherapy and combination therapy, respectively[10]. In COMMIT, respiratory infections occurred in 46% of patients treated with combination therapy compared with 41.3% of those treated with IFX (P = NS), although all patients also received an induction course of corticosteroids which may have contributed to these very high infection rates[12]. Despite these reassuring findings it must be emphasized that follow-up of these trials was relatively short (generally limited to 52 wk), and they were underpowered to detect uncommon opportunistic infections. Retrospective observational studies have reported conflicting infectious complication rates in anti-TNF monotherapy compared with combination therapy. Osterman and colleagues found an increased rate of opportunistic bacterial and fungal infections (HR = 2.64; 95%CI: 1.21-5.73) and herpes zoster (HR = 3.16; 95%CI: 1.25-7.97) amongst 577 patients who "stepped up" to ADA or IFX from IMs (92% thiopurines) over a median follow-up of 1.4-1.7 years, but no increase in the rate of serious infections amongst combination therapy compared with anti-TNF monotherapy[27]. Other studies have shown no increase in infections amongst combination therapy compared with anti-TNF monotherapy[28]. Despite these conflicting data on infection rates, an unequivocal signal from randomized controlled trials and observational studies is that corticosteroids impart a significant additive infective risk for both anti-TNF monotherapy and combination therapy exposed patients[29,30].

MALIGNANCY

It is accepted that thiopurines are associated with an increased risk of non-melanoma skin cancer, (NMSC) (basal cell carcinoma and squamous cell carcinoma) in post-transplant recipient patients[31]. Three large observational studies have demonstrated that thiopurine therapy confers a 4-6 fold increase in NMSC amongst patients with IBD and that this risk remains elevated compared to age-matched thiopurine naïve

patients with IBD even after stopping thiopurines[32-34]. In IBD there are no well-designed studies assessing the risk of NMSC in anti-TNF monotherapy, primarily because of confounding due to prior or concomitant thiopurine exposure. A meta-analysis of anti-TNF monotherapy use amongst patients with rheumatoid arthritis demonstrated an increased risk of NMSC (1.45, 95%CI: 1.15-1.76)[35]. In a nested case-control claim database amongst 3288 matched IBD patients, (3288 NMSC matched to 12945 controls) sub-group analysis of patients with ⩾ 1 year drug use demonstrated the greatest risk amongst combination thiopurines and anti-TNF, (adjusted OR = 3.89, 95%CI: 2.33-6.46) compared to thiopurine monotherapy (adjusted OR = 2.72, 95%CI: 2.27-3.26) and anti-TNF monotherapy (adjusted OR = 1.63, 1.12-2.36)[34]. Amongst patients with less than 12 mo anti-TNF use there was no association with NMSC. A pooled analysis of 1594 CD patients who participated in the landmark RCTs of ADA demonstrated no increased risk of NMSC in ADA monotherapy, compared with an increased risk of NMSC, and other malignancies, in thiopurine combination therapy (adjusted RR = 4, 95%CI: 1.23-13.0)[36]. Taken together, these results suggest that combination therapy increases the risk of NMSC above and beyond the risk of both thiopurine and anti-TNF monotherapy. Despite an apparent increased risk of melanoma amongst patients with IBD[34,37], thiopurine use does not seem to increase the risk further[34]. Anti-TNF therapy, in contrast, appears to double the risk of melanoma[34]. Similar associations between anti-TNF use and melanoma in RA have been observed[35,38,39]. As with NMSC, drawing firm associations between anti-TNF monotherapy exposure and melanoma risk are limited by current or past exposure to IMs.

Determining the influence of IM monotherapy *vs* combination therapy on lymphoma development is difficult due to the relatively uncommon occurrence of this event and the short follow-up period of RCTs. Pooled data from 7054 IBD patients from 11 RCTs, (IFX, ADA, certolizumab and golimumab) followed for 1 year, showed no cases of lymphoma amongst anti-TNF treated patients, compared to 3 placebo arm patients, (although 2 of these had received induction with anti-TNF)[40]. Other pooled analyses have demonstrated an increased risk of lymphoma with combination therapy, however these have not detected cases of lymphoma amongst those treated with anti-TNF monotherapy. This limits the strength of conclusions on the risk of lymphoma development between the two treatment strategies. Accordingly, data from large population-based observational cohort studies must be considered. In CESAME, a prospective observational cohort study of 19 486 IBD patients, the risk of lymphoma was higher amongst patients using thiopurines in combination with anti-TNF compared to thiopurines alone, [standardized incidence ratio, (SIR) = 10.2, 95%CI: 1.24-36.9, $P < 0.04$] *vs* 6.53, 95%CI: 3.48-11.2, $P < 0.0001$, respectively)[41]. Anti-TNF monotherapy did not increase the risk of lymphoma, (SIR = 4.5, 95%CI: 0.6-16.4, $P = 0.1$). Similarly a retrospective cohort study of 36891 Veteran Affairs UC patients, of which 4734 were treated with thiopurines for one year found an increased risk of lymphoma amongst thiopurine users (HR = 4.2, 95%CI: 2.5-6.8, $P < 0.001$)[42]. Subgroup analysis demonstrated a non-significant increased incidence rate ratio, (IRR) amongst thiopurine/IFX combination therapy (IRR = 3.84, 95%CI: 0.8-44.2) compared with thiopurine monotherapy (IRR = 3.6, 95%CI: 2.2-6.0) however only 1 case of lymphoma was diagnosed in the combination group, implying this study was underpowered to detect a true difference. The findings from other studies have been conflicting[27,43-48]. In general, observational studies and meta-analyses have shown that combination therapy increases the risk of lymphoma, however the magnitude of this risk is similar to that seen with IM monotherapy.

UNANSWERED QUESTIONS REGARDING THE OPTIMIZATION OF IMMUNOMODULATORS WHEN USED AS COMBINATION THERAPY

Which immunomodulator should be used - thiopurines or methotrexate?

The evidence as to which IM, thiopurines or MTX, to choose in combination therapy is limited, although there are more data relating to the use of thiopurines. Randomized controlled trials (RCTs) in both CD (SONIC)[10] and UC (SUCCESS)[11] demonstrate superiority of thiopurine-based combination therapy over anti-TNF monotherapy. In contrast, combination therapy with MTX has not been proven to be superior to monotherapy in CD (COMMIT)[12], and there are a lack of high quality data to support the use of MTX in UC when given as monotherapy, with no combination therapy data available[49]. However, given differing trial designs and endpoints, direct comparison of these RCTs must be interpreted with caution.

The benefits of adding an immunomodulator to anti-TNF therapy, even in patients who have previously failed immunomodulators, are presumably due to both a reduction in immunogenicity with a resultant increase in serum anti-TNF levels, and also a direct effect in reducing disease activity. Both thiopurines and MTX have beneficial effects on the pharmacokinetics of anti-TNF agents when used in combination therapy. In a retrospective, single-centre study of 174 CD patients treated with episodic IFX, AZA and MTX were equally effective in preventing immunogenicity (antibodies to IFX, (ATIs) 48% in AZA group *vs* 44% in MTX group, P = NS) and infusion reactions (18% *vs* 14% in AZA and MTX groups respectively, P = NS), and in increasing serum IFX levels (6.15 μg/mL *vs* 5.65 μg/

mL in AZA and MTX groups respectively, *P* = NS)[50]. The presence of ATI was associated with a shorter duration of response in patients not taking IM (median 11.7 wk) as compared to those taking IM (median 13.8 wk, *P* = 0.006) although numbers were small. In SONIC, patients on combination therapy with AZA had significantly higher IFX levels than monotherapy patients at week 30 (3.5 μg/mL *vs* 1.6 μg/mL, *P* < 0.0001)[10]. In the COMMIT study patients on combination therapy with MTX had lower rates of ATI formation than monotherapy patients (4% *vs* 20%, *P* = 0.01) and a trend to higher serum IFX levels (6.35 μg/mL *vs* 3.75 μg/mL, *P* = 0.08)[12].

Another advantage of thiopurines is the oral route of administration, compared to MTX, where only parenteral monotherapy in CD has been consistently demonstrated to be effective[51,52]. If used in therapeutic doses in combination therapy, presumably parenteral MTX is the best option. However if used primarily to reduce immunogenicity then rheumatologic data suggests that low dose oral MTX may be adequate. Published only in abstract form, it was demonstrated that the addition of MTX to maintenance ADA increased ADA levels from 5 μg/mL to between 8-9 μg/mL[53]. More recently in the CONCERTO trial 395 RA patients were randomized to open-label ADA 40 mg alternate weekly, and blinded oral MTX at doses or 2.5, 5, 10 and 20 mg weekly. ADA serum concentrations increased with increasing MTX doses up to 10 mg weekly, above which there was no dose response. Anti-adalimumab antibody prevalence was also similar between the 10 and 20 mg MTX groups, suggesting that in RA patients 10 mg MTX orally weekly is the correct dose to optimize ADA pharmacokinetics[54]. Whether these data are applicable to IBD is unknown. Similarly, thiopurines have consistently been shown to increase serum anti-TNF levels when given as combination therapy[10,55], although there are no data delineating an optimal weight-based thiopurine dose needed to achieve maximal serum anti-TNF concentrations.

Another consideration in the choice of concomitant immunomodulator is the small, but real, increased risk of lymphoma associated with thiopurines in IBD. The most recent meta-analysis of both population and referral-based IBD studies demonstrated a SIR of lymphoma of 4.92 (95%CI: 3.10-7.78) amongst thiopurine-exposed patients. The risk was highest amongst males currently receiving thiopurines for at least one year[48]. A similar increased magnitude of risk has been demonstrated in other recent population-based studies and meta-analyses[41,44]. Of particular concern is the association between thiopurine use and hepatosplenic T cell lymphoma (HSTCL), especially in young males under 35 years of age[56]. By contrast there are no studies showing an increased risk of lymphoma with MTX use in IBD, although it must be recognized that this is largely due to a lack of data rather than there being studies definitively showing no association. Studies in rheumatoid arthritis show conflicting data as to whether MTX use is associated with an increased lymphoma risk, either as monotherapy or when combined with anti-TNF agents[57-59]. In considering these data it would seem reasonable to consider MTX as the immunomodulator of choice when lymphoma risk is highest, such as in young males, whereas for other patients the benefits of thiopurines will usually outweigh the small lymphoma risk. Finally MTX is teratogenic and is contraindicated during pregnancy. Due to its long half-life it is recommended to stop MTX 3-6 mo pre-conception in females[60]. Its effects on male fertility and spermatogenesis are controversial; some experts recommend withdrawal in males 3 mo prior to trying to conceive[60].

When should immunomodulators be commenced when used as combination therapy?

The SONIC study demonstrated in a randomized controlled trial that clinical and endoscopic remission occurs most frequently when immunomodulators and IFX are commenced simultaneously in treatment-naïve patients[10]. Pharmacokinetic data from observational single-centre studies has subsequently emerged to support this practice.

In a retrospective study of 217 patients on anti-TNF therapy (108 IFX, 109 ADA) concomitant IMs improved pharmacokinetic outcomes for patients on IFX (83.1% thiopurines, 16.9% MTX), but not ADA (83.3% thiopurines, 16.7% MTX). For IFX, trough levels were significantly higher in the combination therapy group compared to monotherapy patients (7.5 μg/mL *vs* 4.6 μg/mL, *P* = 0.04), while for ADA no difference was seen (13.1 μg/mL *vs* 11.5 μg/mL respectively, *P* = 0.5). Similarly, combination therapy patients were less likely to have ATIs than monotherapy patients for IFX (5.7% *vs* 29.8%, *P* = 0.001), but not ADA (17.2% *vs* 21.6%, *P* = 0.6). Regarding the timing of introduction of the IM, IFX patients in whom IMs were started at the same time as the anti-TNF were less likely to develop ATIs than patients in whom IMs were started later (2.4% *vs* 18.2%, *P* = 0.04); again no difference was seen in ADA patients. Interestingly, there was no association between IM dose and IFX trough levels, and in fact counter-intuitively patients with suboptimal IM doses had higher trough levels (9.81 *vs* 5.36, *P* = 0.02). This study suggests that immunogenicity occurs early in the treatment course of anti-TNFs and that perhaps a lower dose of IM may be sufficient to prevent anti-drug antibody formation and optimize trough levels[61]. It is important to note that this pharmacokinetic study did not assess clinical outcomes, hence it is unclear whether the favorable effect of combination therapy on improving drug levels and reducing ATIs conferred a clinical benefit. Consistent with these results, in a prospective observational study of 125 patients treated

with IFX (98 CD, 27 UC), 46% of patients developed ATIs. Of these, 90% of patients who developed permanent ATIs did so within 12 mo of starting IFX, whilst transient, and clinically non-significant, antibodies developed at any time during therapy (P < 0.001). Patients on combination therapy had a longer ATI-free survival compared to monotherapy patients (P = 0.003, log rank test)[17]. Low IFX trough levels and high ATI titers were significantly more prevalent amongst patients with clinical loss of response, P < 0.001. These data therefore also demonstrate that IMs are most effective at reducing immunogenicity in the first 12 mo of anti-TNF therapy, suggesting that the two classes of therapy should be commenced simultaneously.

What dose of immunomodulator should be used when used as combination therapy - are lower doses equally effective and safer?

To date most studies of combination therapy have used full weight-based thiopurine doses (AZA-2.0-2.5 mg/kg per day, MP-1.0-1.5 mg/kg per day), with or without further dose-optimization aiming for therapeutic metabolite levels [6-thiogunanine nucleotide, (6-TGN) 235-450 pmol/8 × 10^8 RBC]. However, more recently, definite signals of thiopurine toxicity have been confirmed in large population-based studies, in particular the risk of infections, NMSC and lymphoma[32,41]. Of these adverse events, infection risk is definitely dose-dependent, however most population-based studies of NMSC and lymphoma risk have not included thiopurine doses in their analyses[32,48]. This raises the question of whether lower thiopurine doses can be used in combination therapy with equal efficacy and pharmacokinetic benefits on serum anti-TNF levels, and presumably, less toxicity. Recent retrospective and observational studies have explored the effect of thiopurine dose on outcomes when used in combination therapy, analyzing by mg/kg daily doses or surrogate measures of 6-TGN levels and changes in mean corpuscular volume (MCV) in thiopurine-treated patients.

In the Dutch retrospective study assessing pharmacokinetic outcomes of combination therapy (predominantly with thiopurines) there was no correlation between IM dose and anti-TNF levels, suggesting that lower IM doses in combination therapy may be equally effective[61]. More recently, in a single centre cross-sectional study of 72 patients (45 CD, 27 UC) on combination therapy with scheduled maintenance IFX and thiopurines, thiopurine metabolite levels were correlated with IFX levels and ATIs. There was a moderate correlation between 6-TGN concentrations and IFX levels (rho - 0.53, P < 0.0001). The 6-TGN cut off that best predicted higher IFX levels was 125 pmol/8 × 10^8 RBCs (AUROC - 0.86, P < 0.001). Patients with 6-TGN levels below this cut off had IFX levels similar to patients on monotherapy (4.3 μg/mL *vs* 4.8 μg/mL, P = 0.8). Similarly, patients with 6-TGN levels below this threshold were more likely to have ATIs (OR = 1.3, 95%CI: 2.3-72.5, P < 0.01). These results provide the first signal that lower thiopurine doses, as measured by metabolite levels, may be equally effective as therapeutic doses in optimizing serum anti-TNF levels, however they must be interpreted with caution. The primary endpoint was IFX levels, with mucosal healing as a secondary endpoint, and IFX levels of > 8.3 μg/mL were associated with mucosal healing. When dichotomized above and below this cutoff, a mean 6-TGN level of 223 pmol/8 × 10^8 RBCs was required to achieve an IFX level of 8.3 μg/mL, compared to mean 6-TGN levels of 128 pmol/8 × 10^8 RBCs for IFX levels < 8.3 μg/mL (P < 0.001). Similarly, undetectable *vs* detectable ATIs were associated with mean 6-TGN levels of 117 pmol/8 × 10^8 RBCs and 193 pmol/8 × 10^8 RBCs respectively (P = 0.024). Therefore, while a 6-TGN level of 125 pmol/8 × 10^8 RBCs best predicted increased IFX levels, very similar 6-TGN levels were associated with a lack of mucosal healing and the development of ATIs - this disparity may in part be explained by the high IFX cut off of 8.3 μg/mL that was used, for which sensitivity and specificity were only moderate (71% and 73% respectively)[62]. Similar findings were observed in a single centre cross-sectional study of 269 IBD patients treated with IFX who underwent TDM with a drug-tolerant mobility shift assay[63]. Patients co-treated with AZA/MP, [n = 99 (37%)] and MTX [n = 32 (12%)] were more likely to have therapeutic IFX levels than those on monotherapy, (P = 0.05 and P = 0.04 for thiopurines and MTX, respectively). Regression analysis did not demonstrate a relationship between AZA dose and drug levels (P = 0.88) nor was an association seen between weight based dose (mg/kg) and drug levels when analysed by quartiles (P = 0.87).

The change in MCV with thiopurine therapy has been correlated with 6-TGN levels, with a delta MCV of at least 7 fL being associated with therapeutic 6-TGN levels and improved clinical outcomes[64,65]. A post hoc analysis of the SONIC study [which included only patients with normal thiopurine methyltransferase, (TPMT) activity] investigated the relationship between the change in MCV (dichotomized to above and below 7 fL) and outcomes in patients receiving combination therapy with AZA and IFX. An increase in MCV of at least 7 fL was associated with mucosal healing at week 26 (75% *vs* 47.1% if delta MCV < 7 fL, P = 0.02) and IFX levels > 3.0 μg/mL (68.4% *vs* 38.8% if delta MCV < 7 fL, P = 0.003). On multivariate analysis, delta MCV > 7 fL was associated with mucosal healing (OR = 3.86, 96%CI: 1.05-14.19, P = 0.04). Interestingly, patients with a delta MCV > 7 fL had less infectious adverse events (26.5% *vs* 49.2% if delta MCV < 7 fL, P = 0.008). No correlation between changes in MCV and mg/kg thiopurine doses was performed and thiopurine

metabolites were not measured[66]. These results represent progress in optimizing thiopurines when used in combination therapy, although the optimal mg/kg dose, or surrogate measure of efficacy, remain to be determined.

Similarly, for MTX there are few data to guide clinicians as to the optimal dose, and route, to use in combination therapy with anti-TNF agents in IBD. In rheumatoid arthritis, 10 mg MTX orally weekly was the optimal dose to increase serum adalimumab levels in a MTX dose-escalation study[54]. In the COMMIT study subcutaneous MTX was commenced at 10 mg weekly and increased to 25 mg weekly by week 5, with the mean MTX dose at week 50 being 22.3 mg. At this dose, combination therapy patients compared to monotherapy patients had less ATIs (4% *vs* 20%, $P = 0.01$), numerically higher IFX trough levels (6.35 μg/mL *vs* 3.75 μg/mL, $P = 0.08$) and were more likely to have detectable IFX trough levels (52% *vs* 44%, $P = 0.84$). Even at this high dose, there was no difference in adverse event rates between the two groups[12]. More recently, in a single referral-centre retrospective study of combination MTX and anti-TNF therapy, outcomes were compared between patients on low dose (< 12.5 mg weekly) and high-dose (15-25 mg weekly) MTX. 73 IBD patients with active disease were included (CD-54, UC-16, indeterminate colitis - 3), of which 71% received high-dose and 29% low-dose MTX. The anti-TNF was ADA in 49% of patients, IFX in 40% of patients and certolizumab in 11% of patients, and MTX was given orally in 75% of patients. 46 of 73 (62%) patients went into remission and were followed and included in the primary analysis of duration of remission maintenance. High-dose MTX combination therapy patients were less likely to relapse (log-rank test, $P < 0.01$), and although rates of adverse events (33% *vs* 12%, $P = 0.13$) and discontinuations (14% *vs* 6%, $P = 0.34$) were higher in the high-dose MTX group, these differences did not reach significance. There were no differences when analyzed by the anti-TNF used in combination therapy (log-rank test, $P = 0.58$), diagnosis (log-rank test, $P = 0.78$), or mode of MTX administration (log-rank test, $P = 0.56$). Therapeutic drug monitoring was not performed[67].

Although a lower dose of concurrent IM would be hoped to be safer, in particular resulting in fewer infections and malignancies, there are few data to support this assumption. Studies amongst non-IBD populations have found a relationship between rates of malignancy and total thiopurine dose, thiopurine metabolite levels and TPMT activity[68-70]. Caution must be exercised before extrapolating these findings to the setting of combination therapy in IBD. Thiopurines are associated with increased infections, and viral infections in particular, (as outlined above) although a post-hoc analysis did not find a difference in infection risk between patients on high dose *vs* low dose thiopurines[27]. Similarly, the risk of NMSC and lymphoma associated with thiopurines has never been demonstrated to be dose-dependent in IBD, however most studies addressing these questions have not included IM dose[32,44,48]. From these data, which are mainly retrospective or post hoc analyses, it is not possible to conclude whether a lower dose of concurrent IM is equally efficacious and safer in combination therapy. For thiopurines, "therapeutic" 6-TGN levels were required to achieve IFX levels associated with mucosal healing, while a rise in MCV of > 7 fL may be a useful surrogate target if replicated in other studies. For MTX, unlike rheumatologic studies where lower doses appear adequate to maximize anti-TNF levels, in IBD higher doses (15-25 mg weekly) were required to maintain remission. Therefore until well-designed prospective studies prove otherwise, using full doses of IMs as combination therapy appears to be the best option for clinicians.

CAN IMMUNOMODULATORS BE STOPPED AT ANY TIME WHEN USED IN COMBINATION THERAPY?

In combination therapy patients with a high risk of adverse events to continuing therapy and a low risk of disease relapse on treatment withdrawal, cessation of therapy can be considered. Either the anti-TNF or the IM can be stopped, although relapse rates after IM withdrawal are generally lower than relapse rates after anti-TNF discontinuation, making IM withdrawal the more logical strategy[71]. Another rationale for stopping the IM comes from recent data showing that the risk of malignancy with thiopurines, and lymphoma in particular, is associated with the duration of therapy and reduces, or even normalizes, after IMs are ceased. In the CESAME cohort the hazard ratio for lymphoma was 5.28 (95%CI: 2.01-13.9, $P = 0.0007$) for those continuing thiopurines, but became insignificant (HR = 1.02, 95%CI: 2.01-13.9, $P = 0.98$) after they were ceased[41]. More recently in a retrospective cohort study of 36,891 veterans with UC the hazard ratio for developing lymphoma in patients on thiopurines was 4.2 (95%CI: 2.5-6.8, $P < 0.0001$), but reduced to 0.5 (95%CI: 0.2-1.3, $P = 0.17$) after thiopurines were discontinued[42]. In the most-recent meta-analysis combining 18 population-based and referral-centre studies lymphoma risk became significant after 1 year of thiopurine exposure. Amongst population studies standardized incidence ratios (SIR) were increased amongst current (SIR = 5.71, 95%CI: 3.72-10.1), but not former users (SIR = 1.42, 95%CI: 0.86-2.34)[48]. Similar trends of a reduction in malignancy risk after cessation of therapy have been demonstrated in some thiopurine-associated NMSC cohorts[32,72].

The first well-designed, albeit open-label, study of IM withdrawal (the IMID Study) came from the Leuven group in which 80 CD patients in remission on combination therapy for at least 6 mo were randomized to continue or stop IM therapy, with both

groups continuing scheduled maintenance IFX for 2 years. There was no difference in the primary endpoint of patients requiring a decrease in IFX dosing interval (60% in patients continuing IMs *vs* 55% in patients stopping IMs, $P = 0.65$) or stopping IFX (27.5% *vs* 22.5% respectively, P = NS). Mucosal healing rates were also similar between groups. However patients continuing on IMs had significantly higher trough IFX levels (2.87 μg/mL *vs* 1.65 μg/mL, $P < 0.0001$) and correspondingly lower levels of CRP (1.6 mg/L *vs* 2.8 mg/L, $P < 0.005$), suggesting the possibility of differing outcomes between groups over a longer period of follow up[55]. In a single-centre observational study of 48 CD patients on combination therapy for at least 6 mo in whom AZA was stopped, survival without IFX failure was 85% at 12 mo and 41% at 24 mo. Predictors of IFX failure were a duration of combination therapy less than 27 mo (HR = 7.46, 95%CI: 1.64-33.85, $P = 0.01$) and presence of inflammation at the time of IM withdrawal (CRP > 5 mg/L, HR = 4.79, 95%CI: 1.52-15.10, $P = 0.008$, and platelet count > 298 (HR = 4.75, 95%CI: 1.28-17.57, $P = 0.02$)[73]. More recently, in another single-centre, retrospective study the Leuven group assessed the effect of IM withdrawal on IFX trough levels and immunogenicity. Of 158 patients on combination therapy for at least 6 mo (median 13 mo), IM were withdrawn in 117 patients who were followed for a median of 29 mo. Of patients stopping IMs 38% required an increase in IFX dosing interval and 18% stopped IFX. However IFX trough levels were unchanged before and after IM withdrawal (3.2 μg/mL *vs* 3.7 μg/mL respectively, $P = 0.70$). Low IFX trough levels and high CRP at the time of IM withdrawal, and previous IFX dose-escalation prior to IM withdrawal were predictors of subsequent IFX monotherapy failure. Interestingly, no patients with an IFX trough level > 5 μg/mL at the time of IM withdrawal relapsed during the follow up period[74]. From these three studies it can be concluded that the lowest risk of relapse is in patients who are in deep remission (clinical remission and normalized biomarkers including mucosal healing), with good anti-TNF drug levels, after a prolonged period of combination therapy (ideally at least 12 mo) before IMs are withdrawn. Patients with active disease who withdraw IM are more likely to flare and subsequently require optimization of treatment.

Hopefully the upcoming international BIOCYCLE study, which aims to compare outcomes of treatment cycles in patients on combination therapy to outcomes when either the anti-TNF or IM is withdrawn will provide further clarification of the safety of de-escalation strategies in individual patients.

Of relevance to the issue of de-escalation of therapy, two small recent studies have shown that in patients losing response to anti-TNF monotherapy the re- addition of an IM can overcome immunogenicity and recapture response in some patients. In a small series of 5 patients losing response to IFX due to immunogenicity the addition of an IM (thiopurines in 3 patients, MTX in 2 patients) was successful in overcoming ATIs, increasing serum IFX levels and restoring clinical response in all patients[75]. Similar results were demonstrated when thiopurines were added to five patients failing ADA monotherapy, all of whom had previously failed thiopurine monotherapy. Clinical improvement was noted in all patients and repeat endoscopy was performed in four patients, all of whom showed improvement[76].

CONCLUSION

Over the last 15 years there have been great advances in the understanding of the relative roles IMs and anti-TNFs play in the modern management of IBD. It has become recognized that amongst thiopurine naïve patients, combination therapy is more efficacious than monotherapy with either thiopurines or anti-TNF alone, albeit at an increased risk of adverse events, most important of which are infection and malignancy. However questions remain as to how best to position IM use in those who require treatment with an anti-TNF, particularly in IM failures. Many of these are being addressed as we learn more about the pharmacokinetic relationship between anti-TNF and IM use and clinical outcomes. Combination therapy is associated with higher anti-TNF drug levels and less anti-drug antibody production, especially during the first 12 mo. Higher drug levels, in turn, measured post-induction[77-80] and during maintenance therapy[81-84], are associated with favorable clinical outcomes. Whereas it is tempting to equate the beneficial effects of combination therapy solely to an improvement in anti-TNF pharmacokinetics, it must be recognized that this conclusion is at present intuitive rather than evidence based. Prospective studies are needed that assess differences in efficacy, safety and costs between combination therapy *vs* anti-TNF monotherapy with anti-TNF dose-adjustments to achieve similar drug levels[85]. Further research is also needed to determine the effect of varying thiopurine and MTX doses on anti-TNF pharmacokinetics, incorporating both weight-based and metabolite-based (thioguanine nucleotides and MTX polyglutamates[86], for thiopurines and MTX respectively) dose-optimization strategies.

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TOPIC HIGHLIGHT

2015 Advances in inflammatory bowel disease

Nanomedicine and drug delivery strategies for treatment of inflammatory bowel disease

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Abstract

Crohn's disease and ulcerative colitis are two important categories of human inflammatory bowel disease (IBD). Because the precise mechanisms of the inflammation and immune responses in IBD have not been fully elucidated, the treatment of IBD primarily aims to inhibit the pathogenic factors of the inflammatory cascade. Inconsistencies exist regarding the response and side effects of the drugs that are currently used to treat IBD. Recent studies have suggested that the use of nanomedicine might be advantageous for the treatment of intestinal inflammation because nano-sized molecules can effectively penetrate epithelial and inflammatory cells. We reviewed nanomedicine treatments, such as the use of small interfering RNAs, antisense oligonucleotides, and anti-inflammatory molecules with delivery systems in experimental colitis models and clinical trials for IBD based on a systematic search. The efficacy and usefulness of the treatments reviewed in this manuscript have been demonstrated in experimental colitis models and clinical trials using various types of nanomedicine. Nanomedicine is expected to become a new therapeutic approach to the treatment of IBD.

Key words: Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Nanomedicine; Small interfering RNA; Antisense oligonucleotide

Core tip: Crohn's disease and ulcerative colitis are important categories of human inflammatory bowel disease (IBD). IBD treatment generally involves attempting to inhibit pathogenic factors of the inflammatory cascade. Recent studies suggest that nanomedicine provides advantages over conventional treatments for the treatment of intestinal inflammation because nano-size molecules can effectively penetrate epithelial and inflammatory cells. The efficacy and usefulness of the nanomedicine treatments reviewed

in this manuscript have been validated in experimental colitis models and clinical trials. Nanomedicine is therefore expected to become a new therapeutic approach to the treatment of IBD.

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INTRODUCTION

Inflammatory bowel disease (IBD), which primarily refers to Crohn's disease (CD) and ulcerative colitis (UC), is characterized by chronic inflammation of the gastrointestinal tract[1]. Although the etiology of these diseases remains unknown, several factors such as immune imbalance, dysregulation of the host-microbial interaction, and genetic susceptibility are involved in the pathogenesis of IBD[2]. IBD is treated using 5-aminosalicylic acid (5-ASA), corticosteroids, immunosuppressive drugs and anti-tumor necrosis factor α (TNF-α) antibodies (Abs). However, more than one-third of patients do not respond fully to these therapies. While the efficacy of these drugs decreases over time, the risks of infections and cancer associated with their use are increasing[3-5]. Seventy cases of mycobacterial infections were reported in patients receiving anti-TNF-α Abs by 2001, and the incident rate was more than 10 times the expected background rate[4]. Several studies have shown an association between anti-TNF-α Abs and cancers such as non-Hodgkin's lymphoma (NHL) and cutaneous malignancies. A standardized incidence rate of NHL in over 16000 IBD patients was reported to be 5.5 (95%CI: 4.4-6.6)[6], and the odds ratio of developing cutaneous malignancies was reported to be 2.07 (95%CI: 1.28-3.33)[7].

The medical applications of nanotechnology include the use of nano-particles (NPs) in imaging, pathological diagnosis, and drug delivery. Nanomedicine is a promising tool for the targeted delivery of drugs to specific tissues[8]. Several studies have shown that drugs that are delivered using NPs have advantages over conventional drugs, yielding more effective targeting, greater availability in diseased tissues, and fewer adverse effects. Thus, NPs represent an ideal drug delivery system for the treatment of IBD. NPs not only improve the efficacy of conventional drugs but also aid in the development of new therapeutic drugs. For example, 5-ASA, a conventional drug, is the drug most often studied when attempting to improve delivery systems because it acts only topically. Luminal pH and sustained release are important for delivery systems[9]. Recently, the use of NPs as delivery vehicles for 5-ASA, corticosteroids, and immunosuppressive drugs has been shown to result in greater therapeutic effects in experimental colitis models of IBD compared to standard formulations[10].

Anti-TNF-α Abs, such as infliximab, adalimumab, certolizumab, and golimumab, have proven efficacious against IBD. However, anti-TNF-α Abs therapies require parenteral administration at relatively high doses to achieve their therapeutic effect in the inflamed intestine, increasing the risk of adverse effects, such as lymphoma, infections (especially tuberculosis reactivation), lupus-like syndrome and the generation of anti-infliximab Abs[11]. Strategies that blockade TNF-α effects are needed to improve the safety of these biological therapies. Small interfering RNA (siRNA) and antisense oligonucleotides (ASOs) are candidates for IBD treatment due to their ability to locally neutralize TNF-α.

Biological treatment strategies for IBD involve the neutralization of proinflammatory cytokines, the use of anti-inflammatory cytokines and the inhibition of neutrophil adhesion or T cell signaling. The biological delivery of drugs to inflamed intestines remains a crucial challenge in the current treatment of IBD; therefore, combining siRNA, ASO, and anti-inflammatory molecules with nanotechnology-based drug delivery methods represents a valuable therapeutic approach, and some ASO strategies are already undergoing clinical trials. In this review, we focus on novel therapeutic approaches using nanotechnological systems, such as those that combine siRNA, ASO, and anti-inflammatory molecules with a delivery system.

SIRNA THERAPIES

Gene silencing *via* RNA interference (RNAi) is a candidate treatment for IBD. siRNA, usually comprising 20-25 bp double-stranded nucleotides, is a powerful tool for post-transcriptionally silencing gene expression and interferes with the expression of specific genes. siRNA directed against proinflammatory cytokines might be useful in treating intestinal inflammation. However, the low penetration of siRNA across cell membranes is a major obstacle for siRNA therapy. To overcome this problem, various delivery systems have been developed to deliver siRNA to intestinal tissue (Table 1).

TNF-α siRNA therapies using a delivery system

Neutralization of TNF-α Abs was the first biological strategy used in clinical practice and was more effective at treating IBD than conventional therapies[12]. However, serious infections and side effects were reported, including infusion reactions and the formation of antibodies against TNF-α[13]. Recently, several groups have attempted to drive TNF-α gene silencing directly into inflammatory sites in experimental colitis models. Here, we describe six delivery systems that have been used with TNF-α siRNA for the treatment of

Table 1 Small interfering RNA therapies

Target gene	Delivery system	Administration	Ref.
TNF-α siRNA therapy with delivery system			
TNF-α	TKN	Oral	[14]
TNF-α	PEI-PVA	Oral	[15]
TNF-α	NiMOS	Oral	[16]
TNF-α	OMe-P	Oral	[17]
TNF-α	TPP-PPM	*Ex vivo*	[18]
TNF-α	Fab'-bearing PLA-PEG	Oral	[19]
siRNA therapy targeting other molecules with delivery system			
CyD1	Abs to β7 integrin	Intravenous	[22]
TNF-α/CyD1	NiMOS	Oral	[23]
Map4k4	β1,3-D-glucan shell	Oral	[24]
CD98	scCD98-functionalized	Oral	[25]

SiRNA: Small interfering RNA; TKN: Thioketal nanoparticle; PEI-PVA: Polyethyleneimine/polyvinyl alcohol; NiMOS: Nanoparticles-in-microspheres oral system; OMe-P: 2'-O-methyl and propanediol modification; TPP-PPM: Mannosylated bioreducible cationic polymer/sodium triphosphate; Fab'-bearing PLA-PEG: Polylactic acid-polyethylene glycol copolymer/Fab' portion of the F4/80 Ab; scCD98-functionalized: Chitosan-alginate hydrogel/single-chain CD98 Abs.

experimental colitis.

Thioketal nanoparticles (TKNs) were formulated from a poly-(1,4-phenyleneacetone dimethylene thioketal polymer and selectively degraded by reactive oxygen species (ROS). When TNF-α siRNA/TKN was delivered orally, siRNA was released from TKNs in response to abnormally high levels of specific ROS at sites of intestinal inflammation. Orally administered TNF-α siRNA/TKN protected against dextran sodium sulfate (DSS)-induced colitis and effectively decreased TNF-α mRNA levels at sites of intestinal inflammation[14].

TNF-α siRNA/polyethyleneimine (PEI) was loaded into polylactide (PLA) (NP matrix) and then covered with polyvinyl alcohol (PVA) to form NPs, which were efficiently taken up by inflamed macrophages, thus inhibiting TNF-α secretion by the macrophages *in vitro*. The oral administration of TNF-α siRNA/PEI-PVA in lipopolysaccharide (LPS)-treated mouse models reduced the synthesis and secretion of TNF-α in the colon[15].

TNF-α siRNA was encapsulated in type B gelatin NPs and further entrapped in poly (epsilon-caprolactone) (PCL) microspheres to form a nanoparticles-in-microspheres oral system (NiMOS). This system, which exhibits particle sizes smaller than 5 μm, permitted localization in the colon by a controlled degradation of the outer layer and consequent release of the gelatin NPs to the site of inflammation. The oral administration of TNF-α siRNA/NiMOS attenuated DSS-induced colitis[16].

TNF-α siRNA involving 2'-O-methyl and propanediol modifications (TNF-α siRNA/OMe-P) was resistant to nuclease degradation and provided better silencing efficacy *in vitro* than unmodified siRNA. Intrarectally administered TNF-α siRNA/OMe-P significantly ameliorated DSS-induced colitis compared to unmodified and other chemically modified siRNAs[17].

TNF-α siRNA was formulated with mannosylated bioreducible cationic polymer (PPM) and sodium triphosphate (TPP). These NPs exhibited specific affinity to the mannose receptors that were exclusively expressed on the surfaces of the macrophages. TNF-α siRNA/TPP-PPM increased the efficiency of delivery by selectively targeting phagocytic cells at the inflammation site. These NPs reduced the TNF-α level in the intestine of DSS-induced colitis models in an ex vivo study[18].

TNF-α siRNA was loaded into polylactic acid-polyethylene glycol copolymer (PLA-PEG); then, the NPs were grafted to the Fab' portion of the F4/80 Ab (Fab'-bearing) on the surface of the NPs. Fab'-bearing PLA-PEG NPs exhibited improved macrophage-targeting kinetics *in vitro*. Orally administered TNF-α siRNA/Fab'-bearing PLA-PEG attenuated DSS-induced colitis more efficiently than uncovered NPs[19].

siRNA therapies targeting other molecules with delivery system

Other molecules, such as (1) Cyclin D1 (CyD1); (2) a combination of TNF-α and CyD1; (3) mitogen-activated protein kinase kinase kinase kinase 4 (Map4k4); and (4) CD98, have been considered as novel targets for the treatment of IBD using siRNA delivery systems.

CyD1, a key cell cycle-regulating molecule, was upregulated in the epithelial and immune cells of IBD patients, which are implicated in promoting inflammation and epithelial colorectal dysplasia[20,21]. The liposome-based NPs used to target CyD1 siRNA were covered by Abs raised against β7 integrin, a receptor that is specifically present on leukocytes that are involved in intestinal inflammation. CyD1 siRNA/Abs raised against β7 integrin administered intravenously inhibited intestinal inflammatory responses in DSS-induced colitis. Silencing the CyD1 gene decreased the production of Th1 cytokines, such as TNF-α and IL-12[22].

Kriegel *et al*[23] targeted TNF-α and CyD1 using NiMOS[16]. CyD1 siRNA was combined with TNF-α siRNA/NiMOS. The dual silencing effect was more potent than the silencing of TNF-α siRNA alone. This study demonstrated the therapeutic potential of an oral NiMOS-based dual TNF-α and CyD1 gene silencing system for the treatment of IBD in a DSS-induced acute colitis model.

Map4k4 is a mediator of cytokine expression. Map4k4 siRNA was encapsulated in β1,3-D-glucan shells. Glucan has a specific affinity to glucan receptors that are present on macrophages and dendritic cells and is taken into targeted cells by phagocytosis. Orally administered NPs silenced Map4k4 expression in LPS-treated mice, thus protecting the mice from LPS-induced systemic inflammation by suppressing the production of TNF-α and IL-1β[24].

CD98 overexpression on colonic epithelial cells

Table 2 Antisense oligonucleotide therapies

Target gene	Delivery system	Administration	Ref.
ASO			
TNF-α	No	Subcutaneous	[30]
CD40	No	Rectal	[33]
MAdCAM-1	No	Subcutaneous	[34]
STAT3	No	Rectal	[36]
NPY	No	Rectal	[38]
ASO with delivery system			
TNF-α	gal-LMWC	Rectal	[39]
NF-κB	CS-PLGA	Oral	[40]
MIF	SPG	Intraperitoneal	[42]
CD40	nov038	Intravenous	[43]
TNF-α	cKGM	Oral	[44]

ASO: Antisense oligonucleotide; gal-LMWC: Galactosylated low-molecular-weight chitosan; CS-PLGA: Chitosan-modified poly (D,L-lactide-co-glycolide); SPG: Schizophyllan; nov038: Amphoteric liposome; cKGM: Cationic konjac glucomannan phytagel.

and macrophages is involved in the development and progression of IBD[25]. CD98 siRNA was loaded into a chitosan/alginate hydrogel; then, NPs were grafted to single-chain CD98 Abs (scCD98) on the surface of NPs. The scCD98-functionalized CD98 siRNA-loaded NPs were approximately 200 nm in size and exhibited high affinity for CD98-overexpressing cells. These NPs significantly reduced CD98 levels in Colon-26 cells and RAW 264.7 macrophages. Orally administered NPs decreased the severity of colitis in both a T cell transfer mouse model and a DSS-induced colitis model[26].

ANTISENSE OLIGONUCLEOTIDE THERAPIES

Antisense oligonucleotide (ASO) are generally 13 to 25 bases in length; these oligomers are designed to hybridize to mRNA that codes for a targeted protein. ASOs can reduce the abundance of specific RNAs through multiple mechanisms, such as the RNase H-mediated degradation of target RNA, translational arrest, and altered RNA splicing[27]. However, ASOs have a short *in vivo* half-life and poor biological stability because they are rapidly degraded by intracellular endonucleases and exonucleases. Several studies have demonstrated that replacement of the native backbone phosphates with phosphorothioates diminishes the degradation of ASOs by nucleases, thus increasing their stability[28]. Moreover, phosphorothioate oligodeoxynucleotides (ODNs) are highly soluble, easily administered and capable of activating RNase H activity[29]. Phosphorothioate ASOs have been used to target: (1) TNF-α; (2) CD40; (3) mucosal addressing cell adhesion molecule (MAdCAM)-1; (4) signal transducers and activators of transcription 3 (STAT3); and (5) neuropeptide Y (NPY) (Table 2).

ISIS 25302, which is specific for murine TNF-α, is a phosphorothioate ODN that contains methoxyethyl-modified nucleosides on its 5′ and 3′ ends. The methoxyethyl modification increases the affinity of ASOs for targeted mRNA and nuclease resistance. In *in vitro* experiments, ISIS 25302 decreased TNF-α mRNA in a dose- and sequence-dependent manner in a mouse macrophage cell line. ISIS 25302 subcutaneous injection significantly decreased disease activity index scores in mice with both acute and chronic DSS-induced colitis and significantly improved histopathological scores in IL-10-deficient mice[30].

The involvement of CD40 and CD154 in the pathogenesis of IBD is apparent due to their increased expression in the inflamed mucosa of patients and based on the therapeutic effects of anti-CD154 Abs in experimental colitis[31]. Due to their adverse effects, the use of such Abs in patients with IBD might be limited[32]. The rectal administration of CD40 phosphorothioate ASO was used to block CD154/CD40 and effectively interfered with CD154/CD40 interactions and attenuated 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis in rats[33].

The expression of MAdCAM-1 is restricted in gut-associated lymphoid tissues, and its expression is dramatically increased in IBD. MAdCAM-1 phosphorothioate ASOs were injected subcutaneously into TNBS-induced colitis model mice. MAdCAM-1 ASOs significantly suppressed the development of TNBS-induced colitis clinically and histopathologically compared with controls. MAdCAM-1 ASO also reduced the number of α4β7 lymphocytes in the inflamed colonic mucosa[34].

The expression levels of STAT3 are increased in IBD and colitis model mice[35]. STAT3 phosphorothioate ASO was administered by rectal enema during the early phase of TNBS-induced colitis. Administration of STAT3 ASO effectively inhibited STAT3 expression and phosphorylation in the inflamed colonic mucosa of the colitis models, and the rectal administration of STAT3 ASO significantly attenuated intestinal inflammation[36].

In the central nervous system, NPY regulates many physiological functions, including stress. NPY has been shown to play an important role in immune and inflammatory responses[37]. The rectal administration of a NPY phosphorothioate ASO ameliorated DSS-induced colitis in rats, suggesting that NPY plays an important role in modulating inflammation in colitis[38].

ASO DELIVERY SYSTEMS

Naked ASOs are unable to cross cellular membranes and are rapidly degraded *in vivo*. Specialized delivery systems are necessary for the delivery of ASOs to target tissues for therapeutic efficacy. Delivery systems have been reported for various targets, including (1) TNF-α; (2) NF-κB; (3) macrophage-migration inhibitor factor (MIF); (4) CD40; and (5) TNF-α for use in treating IBD (Table 2).

A nano-complex based on galactosylated low-molecular-weight chitosan (gal-LMWC) and TNF-α ASO was developed to target activated macrophages

for use in treating intestinal inflammation. Rectal administration of a TNF-α ASO/gal-LMWC complex resulted in the successful delivery of ASO into activated colonic macrophages and a significant reduction of colonic TNF-α in TNBS-induced colitis. A single injection of TNF-α ASO/gal-LMWC was used to treat TNBS-induced colitis and repeated injections were used to treat T cell-transfer colitis; both treatments significantly ameliorated colitis[39].

Chitosan (CS)-modified poly(D,L-lactide-co-glycolide) (PLGA) NPs were developed and evaluated for use with a NF-κB decoy ODN oral delivery system to treat DSS-induced colitis. NF-κB decoy ODN uptake studies using Caco-2 cells and confocal laser scanning microscopy indicated that CS-PLGA NPs were more effectively taken up by the cells than unmodified PLGA. NF-κB decoy ODN/CS-PLGA improved the stability of ODN against DNase Ⅰ and acidic media, such as gastric juices. Orally administered NF-κB decoy ODN/CS-PLGA significantly attenuated colitis[40].

MIF, which is mainly produced by macrophages, has been shown to have a pathogenic role in IBD[41]. A delivery system for ASO using schizophyllan (SPG), a polysaccharide that belongs to the β-(1-3) glucan family, has been developed. This system has several advantages, enabling the effective suppression of targeted RNA or DNA; the SPG complex is stable *in vivo*, and the SPG complex is effectively taken up into macrophages by phagocytosis through Dectin-1. The intraperitoneal injection of MIF ASO/SPG complex effectively suppressed MIF production and significantly ameliorated intestinal inflammation[42].

CD40-CD40L interactions appear to play an important role in the pathogenesis of experimental colitis. CD40 ASO was formulated in amphoteric liposomes (nov038/CD40 ASO). The charge characteristics of amphoteric liposomes facilitate the efficient sequestration of ASO inside the liposomes at low pH and direct the carriers to macrophages and dendritic cells. Delivery of nov038/CD40 ASO is highly cell-specific because it selectively suppresses CD40 on macrophages but not on B-cells. Systemic administration of nov038/CD40 ASO effectively treated TNBS-induced colitis and prevented its development[43].

TNF-α ASO NPs were constructed using cationic konjac glucomannan (cKGM), phytagel and TNF-α ASO. This DDS enabled the spontaneous release of an ASO/cKGM nano-complex from the phytagel scaffold into the colon lumen, where the ASO was transferred into colonic macrophages *via* receptor-mediated phagocytosis. Orally administered TNF-α ASO NPs significantly attenuated DSS-induced colitis[44].

ASO THERAPIES IN CLINICAL TRIAL

Accumulating evidence has suggested that ASOs can be used to inhibit specific targets, such as (1) NFκB-p65; (2) intercellular adhesion molecule (ICAM)-1; and (3) Smad7 in experimental colitis models; this research has led to clinical trials in IBD patients.

NF-κBp65 ASO

NF-κB is a member of a family of transcription factors that regulate the promoters of several genes, the products of which are involved in many biological processes[45,46]. In TNBS-induced colitis and IL-10-deficient mice (two murine models of colitis), the p65 subunit of NF-κB was strongly activated and played a role in the up-regulation of pro-inflammatory cytokines[47]. Targeting NF-κBp65 was also effective in treating DSS-induced colitis and TNBS-induced colitis[48,49]. Clinical trials for NF-κBp65 ASO are underway.

Alicaforsen

ICAM-1 is constitutively expressed at low levels in leukocytes and vascular endothelial cells. ICAM-1 was shown to be upregulated in the inflamed colon of IBD patients[50], and neutralizing ICAM-1 Abs and ICAM-1 ASOs attenuated colitis in mice[51,52]. Alicaforsen (ISIS 2302), an RNase H-dependent, 20-base-long phosphorothioate ASO that was designed to inhibit human ICAM-1, was the first ASO used to treat IBD. In a phase Ⅰ clinical trial, intravenous alicaforsen was well tolerated[53]. In 20 active CD patients, alicaforsen was superior to placebo in inducing clinical remission[54]. However, the efficacy of alicaforsen was not confirmed in two double-blind, placebo-controlled, multicenter clinical trials[55,56].

Furthermore, the efficacy of alicaforsen was investigated by administering this drug by rectal enema to patients with mild to moderate left-sided UC[57]. Alicaforsen enema showed promising acute and long-term benefits in UC patients. Individual patient data in a meta-analysis of 200 patients from four phase Ⅱ clinical trials confirmed the efficacy of alicaforsen enema in patients with active UC[58].

Mongersen

The cytokine transforming growth factor (TGF)-β1, which is produced by many mucosal cell types, is able to negatively regulate the activation and function of several immune cell types[59]. The immunoregulatory properties of TGF-β1 are mainly mediated by the Smad pathway[60]. Smad7, an inhibitor of TGF-β1 signaling, is overexpressed in IBD mucosa and purified mucosal T cells. Smad7, which is also inhibited by Smad7 ASO in cells isolated from IBD patients, restored TGF-β1 signaling and enabled TGF-β1 to inhibit cytokine production[61]. Smad7 ASO (mongersen), an RNase H-dependent, 21-base phosphorothioate ASO, has been formulated as a solid oral dose. This formulation is protected by an external tablet coating made of pH (6.6-7.2)-dependent metacrylic acid polymers, enabling the antisense to be released only in the lumen of the terminal ileum and right colon. In a phase Ⅰ study, mongersen was demonstrated

Table 3 Administration of anti-inflammatory mediators

Mediator	Delivery system	Administration	Ref.
IL-10			
IL-10	*L. lactis*	Oral	[65]
IL-10	Gelatin microspheres	Rectal	[66]
IL-10	NiMOS	Oral	[67]
Other anti-inflammatory molecules			
TNF-neutralization	*L. lactis*	Oral	[68]
PHB1	PEI-PVA	Oral	[70]
TFF	*L. lactis*	Oral	[72]
KPV	PLA	Oral	[74]
IL-27	*L. lactis*	Oral	[77]

L. lactis: *Lactococcus lactis*; NiMOS: Nanoparticles-in-microspheres oral system; PEI-PVA: Polyethyleneimine/polyvinyl alcohol; PLA: Polylactide.

to be safe and well tolerated in active CD patients. Mongersen treatment produced a significant decrease in CDAI scores[62]. Furthermore, the efficacy of mongersen for the treatment of active CD patients was evaluated in a double-blind, placebo-controlled, phase Ⅱ trial. This study demonstrated that the treatment of active CD patients with mongersen resulted in significantly higher rates of remission and clinical response compared to placebo[63].

ADMINISTRATION OF ANTI-INFLAMMATORY MEDIATORS

The administration of anti-inflammatory mediators, especially IL-10, represents another biologic strategy for IBD. Several anti-inflammatory mediator candidates have been investigated using experimental colitis models (Table 3).

IL-10 NPs

IL-10 is an anti-inflammatory cytokine that suppresses the T helper 1 immune response and down-regulates macrophages and monocytes. The therapeutic effect of the systemic administration of IL-10 to IBD patients has not been satisfactory[55]. This failure is thought to be due to the delivery of only low concentrations of IL-10 to the intestinal tissues. Moreover, higher doses of systemically administered IL-10 caused adverse effects[64]. Topical therapy using nanotechnology, such as oral and rectal administration, might improve efficacy and safety by localizing the effect of IL-10 to the inflammation site, thus preventing side effects.

The oral administration of genetically engineered IL-10-secreting *Lactococcus lactis* (*L. lactis*) provided in situ synthesis of IL-10, which resulted in a 50% reduction of inflammation in DSS-induced colitis mice and prevented the onset of colitis in IL-10-deficient mice[65].

Recombinant IL-10 was loaded into gelatin microspheres (GMs). Rectal administration of these GMs (GM-IL-10) attenuated colitis in IL-10-deficient mice[66].

NiMOS was formulated with IL-10-expressing plasmid DNA in type-B gelatin NPs. These NPs directed the local transfection of IL-10 plasmid in inflamed intestinal tissues and enhanced IL-10 expression. Orally administered plasmid DNA encoding IL-10/NiMOS suppressed proinflammatory cytokines, consequently attenuating TNBS-induced acute colitis[67].

Other anti-inflammatory molecules delivered using NPs

Other anti-inflammatory molecules, such as (1) TNF-neutralizing nanobodies; (2) prohibitin 1 (PHB); (3) trefoil factors (TFF); (4) the tripeptide Lys-Pro-Val (KPV); and (5) IL-27, were investigated in experimental colitis models and might represent novel candidate therapeutics for the treatment of human IBD.

L. lactis was engineered to secrete monovalent and bivalent murine TNF-neutralizing nanobodies as therapeutic proteins. These therapeutic proteins are derived from fragments of heavy-chain camelid antibodies and are more stable than conventional antibodies. Orally administered nanobody-secreting *L. lactis* significantly reduced inflammation in DSS-induced chronic colitis mice and in IL-10-deficient mice[68].

Genetic restoration of intestinal epithelial PHB1 levels during experimental colitis reduced the severity of the disease by sustaining epithelial antioxidant expression and reducing NF-κB activation[69]. Recombinant PHB/polyethyleneimine (PEI) was loaded into polylactide (PLA) NPs and then covered with polyvinyl alcohol (PVA). The therapeutic potential of this system for restoring epithelial PHB was then examined in a DSS-induced colitis model. The oral administration of PHB/PEI-PVA resulted in increased levels of PHB in colonic epithelial cells and decreased severity of colitis[70].

TFFs are cytoprotective and promote epithelial wound healing and reconstitution of the gastrointestinal tract; thus, TFFs are good candidate therapeutics for use in treating acute colitis[71]. The food-grade bacterium *L. lactis* was engineered to secrete bioactive murine TFF. Oral administration of TFF-secreting *L. lactis* led to the active delivery of TFF at the mucosa of the colon and proved very effective in the prevention and healing of acute DSS-induced colitis and in improving established chronic colitis in IL-10 deficient mice[72].

The anti-inflammatory tripeptide Lys-Pro-Val (KPV)[73] was loaded into polylactide (PLA) nanoparticles and encapsulated into a polysaccharide gel containing alginate and chitosan polymers. NP-KPV was much more effective than free KPV in reducing the inflammatory response induced by LPS in the intestinal epithelia of mice. The effective dose of NP-KPV was 12000 times lower than that of KPV in free solution. Furthermore, NP-KPV demonstrated therapeutic efficiency in treating DSS-induced colitis models[74].

IL-27 has an immunosuppressive role[75,76]. A localized IL-27 delivery system was synthesized in *L. lactis* by incorporating a linker between the two chains

of IL-27; codons and a secretory signal sequence preferred by *L. lactis* (LL-IL-27) were used. LL-IL-27 administration protected against colitis in a T cell transfer model by increasing the production of IL-10. The oral administration of LL-IL-27 might be a more effective and safe therapy for IBD[77].

CONCLUSION

In this review, we provide novel insights into the role of nanomedicine in IBD treatment. ASO, siRNA and anti-inflammatory molecules with drug delivery vehicles generally undergo cellular internalization by paracellular transport or endocytosis into intestinal epithelial cells. Specialized differentiated epithelial cells called M cells are involved in the predominant uptake of nanoparticles in healthy intestinal mucosa. In intestinal inflammation, a loss of mucous-gel layers and the epithelial barrier through enterocyte damage and increased delivery of immune cells to the mucosal tissue have been shown to lead to the preferential accumulation and uptake of nanomedicines by both enterocytes and macrophages[78]. Therefore, the topical therapy of nanomedicine by oral and rectal administration can be effective in treating the inflammation site.

Important factors in targeting the intestine are not only the use of nano-size molecules but also the implementation of additional strategies to enhance drug delivery to inflamed intestinal mucosa and achieve maximal retention time in tissues. As summarized in this review, nanomedicine strategies for IBD treatment have proven effective for the treatment of experimental colitis models; however, further studies on the effects of nanomedicine in human IBD are warranted. Specifically, there is a need for further investigation of the safety and efficacy of nanomedicine in human IBD. Recently, the efficacy of phosphorothioate ASOs was demonstrated in patients with IBD and various types of cancer[79,80]. By accumulating further evidence, clinical applications of nanomedicine will be realized. In the future, locally targeted nanomedicine may provide a tailored treatment for the control of the immune response and the inhibition of inflammation in individual IBD patients.

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TOPIC HIGHLIGHT

2015 Advances in Irritable Bowel Syndrome

Genetic epidemiology of irritable bowel syndrome

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Abstract

Irritable bowel syndrome (IBS) is the most common functional gastrointestinal disorder characterized by presence of abdominal pain or discomfort associated with altered bowel habits. It has three main subtypes - constipation predominant IBS (C-IBS), diarrhea predominant IBS (D-IBS) and IBS with mixed features of both diarrhea as well as constipation (M-IBS). Its pathophysiology and underlying mechanisms remain elusive. It is traditionally believed that IBS is a result of multiple factors including hypersensitivity of the bowel, altered bowel motility, inflammation and stress. Initial studies have shown familial aggregation of IBS suggesting shared genetic or environmental factors. Twin studies of IBS from different parts of world have shown higher concordance rates among monozygotic twins than dizygotic twins, and thus suggesting a genetic component to this disorder. Multiple studies have tried to link single-nucleotide polymorphisms (SNPs) to IBS but there is little evidence that these SNPs are functional. Various molecules have been studied and investigated by the researchers. Serotonin, a known neurotransmitter and a local hormone in the enteric nervous system, has been most extensively explored. At this time, the underlying gene pathways, genes and functional variants linked with IBS remain unknown and the promise of genetically-determined risk prediction and personalize medicine remain unfulfilled. However, molecular biological technologies continue to evolve rapidly and genetic investigations offer much promise in the intervention, treatment and prevention of IBS.

Key words: Irritable bowel syndrome; Single-nucleotide polymorphism; Serotonin; Familial aggregation; Genetics

Core tip: Irritable bowel syndrome (IBS) is believed to result from interplay of several factors including hypersensitivity of the bowel, altered bowel motility, inflammation and stress. Familial aggregation of cases and twin studies underscore the genetic basis of IBS. Different researchers have studied several candidate genes but the evidence so far linking IBS to specific genes is inconsistent and weak. Genome wide association studies that can examine several common genetic variants are needed to design newer drugs and

diagnostic methods.

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INTRODUCTION

Irritable bowel syndrome (IBS) is the most common functional gastrointestinal disorder with a worldwide prevalence rates ranging between 7%-21%[1]. It is a clinical diagnosis that does not require diagnostic testing unless needed to exclude other diagnostic possibilities. It is characterized by presence of abdominal pain or discomfort associated with altered bowel habits. Rome Ⅲ criteria that assess the relationship between abdominal pain or discomfort, stool form and change in bowel frequency is the most accepted criteria used in clinical practice for making a clinical diagnosis[2]. Three clinical types have been recognized based on altered bowel motility and the resulting predominant feature - constipation predominant IBS (C-IBS), diarrhea predominant IBS (D-IBS) and IBS with mixed features of both diarrhea as well as constipation (M-IBS).

The genetic basis of IBS has been suggested by familial aggregation and twin studies. Furthermore, over the last decade, several candidate genes have been identified that are potentially linked to IBS. This review will further elaborate on our current understanding of genetic epidemiology of IBS.

GENETIC EPIDEMIOLOGY OF IBS

Familial aggregation of IBS

Mankind is familiar with IBS for more than a century but its pathophysiology and underlying mechanisms remain elusive. It is traditionally believed that IBS is a result of multiple factors including hypersensitivity of the bowel, altered bowel motility, inflammation and stress. Initial studies have shown familial aggregation of IBS suggesting that genes or shared environmental exposures contribute to the development of IBS (Table 1). In one of the earlier studies by Whorwell *et al*[3] while investigating the non colonic symptoms of IBS, investigators found that 33% of IBS patients had a family member with IBS as compared to only 2% in the control group ($P < 0.0001$). In an interesting study by Levy *et al*[4] it was revealed that children of parents who had IBS made 20% more ambulatory care visits than the children of parents without IBS ($P = 0.0001$). In the same study, researchers also showed that these children who had a parent with IBS made 50% more visits for gastrointestinal symptoms as compared to control group ($P = 0.0001$). In the study by Locke *et al*[5], a survey of people residing in Olmsted county of Minnesota with functional gastrointestinal disorders showed higher odds (OR = 2.3; 95%CI: 1.3-3.9) of reporting a relative with gastrointestinal symptoms among these individuals. Studies by Kalantar *et al*[6] and Saito *et al*[7] are the other examples where relatives of IBS patients and controls were interviewed and it was determined that statistically higher percentage of IBS patients' relatives had IBS as compared to control patients' relatives (17% *vs* 7%, OR = 2.7; 95%CI: 1.2-6.3 and 37% *vs* 16%, $P = 0.002$ respectively). In another study by Saito *et al*[8] besides showing aggregation of IBS cases among family members, lack of any association in spouses was also shown. In a recent large nationwide case cohort study from Sweden, a higher odds ratio of IBS was found in first, second and third degree relatives of IBS patients (OR for first degree relatives: 1.75-1.90, for second degree relatives: 1.10-1.78 and third degree relatives: 1.11)[9]. These studies further support the concept of shared genes or shared environmental exposures.

Similarly, twin studies have also demonstrated the role of genes in IBS. The first twin study from Australia by Morris-Yates *et al*[10] and another study involving Swedish twin pairs by Svedberg *et al*[11] emphasized the genetic basis of IBS. Studies by Levy *et al*[12] and Bengtson *et al*[13] involving 281 twin pairs in United States and 3334 twin pairs in Norway respectively showed higher concordance rate for IBS among monozygotic twins than in dizygotic twins (17.2% *vs* 8.4%, $P = 0.03$; 22.4% *vs* 9.1%, $P = 0.011$ respectively). In the study by Lembo *et al*[14], 986 twin pairs from Minnesota twin registry were surveyed and their results concur with results of the studies from the other countries and showed the higher rate of IBS among monozygotic twins. Though all these twin studies mentioned above underscore the genetic basis of IBS, the study by Mohammed *et al*[15] did not come to the same conclusion and found no difference in concordance rates between monozygotic and dizygotic twins.

CANDIDATE GENE STUDIES AND IBS

Serotonin and IBS

Many investigators have focused on specific candidate genes and whether variation in these genes is associated with IBS. Serotonin (5-hydroxytryptamine, 5-HT) is the most widely studied molecule due to its role in brain-gut axis and abundance in the gastrointestinal tract. Majority of body serotonin is present in gastrointestinal tract where it is synthesized mainly by enterochromaffin cells (EC cells) and some of it by myenteric plexus neurons. Serotonin is a known neurotransmitter and a local hormone in the enteric nervous system. It is released from EC cells in response to variety of stimuli and exerts its local

Table 1 Familial aggregation studies

Authors (Year)	Number of IBS patients	Conclusion
Whorwell *et al*[3] (1986)	100	Significant number of IBS patient had another family member with IBS
Levy *et al*[4] (2000)	373	Children of parents with IBS made more visits for gastrointestinal symptoms
Locke *et al*[5] (2000)	643	People with functional gastrointestinal disorders had higher odds of reporting a relative with similar symptoms
Kalantar *et al*[6] (2003)	181	IBS prevalence was higher among IBS patients' relatives
Saito *et al*[7] (2008)	50	Statistically higher percentage of IBS patients' relatives had IBS
Saito *et al*[8] (2010)	477	50% of IBS patients had at least another relative with IBS
Waehrens *et al*[9] (2015)	51952	Increased risk of IBS among first, second and third degree relatives of IBS patients

IBS: Irritable bowel syndrome.

paracrine effects through serotonin receptors. There are seven serotonin receptors identified so far named as 5-HT1 to 5-HT7. Out of these seven receptors, 5HT1 to 5-HT4 and 5HT7 play a key role in mediating intestinal responses. The effect of serotonin molecule initiated after its binding to serotonin receptor is eventually terminated by its uptake through a serotonin reuptake transporter (SERT)[16].

There is evidence that patients with IBS have defects in serotonergic signaling. Almost four decades ago, it was discovered that patients with IBS have more number of EC cells as compared to controls[17,18]. Since then numerous studies have explored the relationship between EC cells, serotonin and IBS. Study by Bearcroft *et al*[19] demonstrated that patients with D-IBS have higher blood levels of serotonin. Similarly studies by Dunlop *et al*[20] and Atkinson *et al*[21] have shown higher blood levels of serotonin in D-IBS patients and lower blood levels of serotonin in C- IBS patients. These authors also measured the levels of 5-HIAA (5-hydroxyindoleacetic acid), a 5-HT metabolite, in the rectal mucosal biopsy specimens and blood to obtain 5-HT/5-HIAA ratios as a surrogate marker for serotonin turnover. Based on analysis of 5-HT/5-HIAA ratios in blood and mucosal biopsies, serotonin release defects in C-IBS and serotonin uptake defects in D-IBS were suggested. In the search for an explanation to these differences SERT functions, polymorphism of SERT gene has been extensively investigated.

The SERT gene, also known as solute carrier family 6 member 4 (SLC6A4), was mapped to chromosome 17q11.2 by Ramamoorthy *et al*[22] in 1993. Polymorphic loci that affect the expression and function of SERT gene have been identified. There is a GC base pair rich repetitive sequence located at 5' regulatory end of the SERT gene and is labeled as 5-HT transporter linked promoter region (5-HTT LPR). Polymorphism due to deletion or insertion of 44 base pairs in this region resulting in a long (L) and short (S) allele was first discovered by Heils *et al*[23]. Another common polymorphism of SERT gene results from a variable number of 17 base pair repeats (VNTR: variable number tandem repeats) in the intron 2 of gene[24]. VNTR has 4 alleles described with 9, 10, 11 and 12 repeats.

The evidence on the relationship between different genotypes and their phenotypic expression comes from a study conducted by Lesch *et al*[25]. Lesch *et al*[25] found that S/S genotype as compared to other genotypes L/L and S/L had less 5-HT uptake resulting in higher blood levels of 5-HT. Based on studies from Dunlop *et al*[20] and Atkinson *et al*[21] where defects in serotonin uptake were suggested in D-IBS, S/S genotype was expected to be associated with D-IBS. Several researchers have explored the association between IBS and SERT gene since then but have come up with conflicting results. In 2002 for the first time, Pata *et al*[26] demonstrated higher percentage of C-IBS patients to have S/S genotype and D-IBS to have L/S genotype. In the same year Camilleri *et al*[27] investigated the association between SERT polymorphism and response to Alosetron (5-HT3 receptor antagonist) and found higher response rates to the drug in IBS individuals with L/L genotype. According to the study by Lee *et al*[28] in the Korean population there was no association between IBS and SERT gene polymorphism. However, another Korean study by Park *et al*[29] found significantly higher frequency of S/S genotype among patients with D-IBS, contrary to results of study by Pata *et al*[26] mentioned above. Similarly, in the North American study by Yeo *et al*[30] S/S genotype was found to be associated with D-IBS female patients but Saito *et al*[31] found a significantly higher number of S/S genotype with M-IBS patients. In the study involving Indian IBS patients a significant association was found between S/S genotype and C-IBS[32]. In a Chinese study by Li *et al*[33] allele frequency of the L/L genotype was significantly higher in the C-IBS group. In the same study, researchers also demonstrated poor response to Tegaserod (5HT4 partial selective agonist) associated with L/L genotype.

Another polymorphism pertaining to a single nucleotide polymorphism locus, rs25531 that is located immediately upstream of 5-HTTLPR was described by Kohen *et al*[34]. It has two - A and G alleles. Hu *et al*[35] showed that A variant of L allele (designated as L_A) yields higher SERT expression as compared to G variant of L allele (designated as L_G). This implies that L_G (G variant of rs25531 with L allele) actually behaves as the low expressing S allele. With respect to VNTR polymorphism, one study[36] found a significant association between the VNTR polymorphism and IBS but other studies by Yeo *et al*[30], Pata *et al*[26] and Li *et al*[33] did not find any significant association.

To investigate the possible association between IBS subtypes and SERT polymorphism, Van Kerkhoven *et al*[37] conducted a meta-analysis of eight studies in 2007 that found no significant association between SERT polymorphism and IBS subtypes. Another meta-analysis conducted in 2013 that included more recent studies conducted since the first meta-analysis by Van Kerkhoven *et al* in 2007, concluded a positive association between SERT polymorphism and C-IBS[38]. In another meta-analysis from 2013 performed by Areeshi *et al*[39] that included twelve studies comprising 2068 IBS patients, no association was found between SERT polymorphism and IBS overall. However, when the studies were stratified according the country of origin, significant association was found in American and Asian studies. The most recent meta-analysis with the largest sample size, involving 25 studies comprising of 3443 IBS patients found a positive association between SERT polymorphism and IBS but this association was found only in the East Asian population and not in the Caucasian population[40]. One probable explanation for this ethnic difference, as evidenced by this meta-analysis, could be the significantly lower frequency of L allele among the East Asian population as compared to Caucasian controls.

Besides the most widely studied polymorphism involving SERT LPR discussed above, several other polymorphism loci have been explored. These include polymorphisms of gene involving other serotonin receptors - *5-HT2A* gene[41-43] and *5-HT3E* gene[44] and yet again the studies have yielded either conflicting results or need further validation in other ethnic groups.

Tryptophan hydroxylase (TPH), the rate-limiting enzyme in 5-HT biosynthetic pathway has two isoforms - TPH1 and TPH2 encoded by genes on chromosome 11 and 12 respectively. Jun *et al*[45] found no association between TPH1 gene single nucleotide polymorphism and risk of developing IBS, however found significant association with severity as well as number of days with diarrhea in IBS patients. TPH2 gene polymorphism was also tested and shown to be associated with reduced risk of having IBS but statistically this difference was barely significant. Similar to this study by Jun *et al*, no association was found between genotype frequencies and IBS in the study conducted by Grasberger *et al*[46]. But the CC genotype was found to be more prevalent in IBS-D patients in the study. Researchers that investigated the colonic mucosa levels of TPH1 mRNA in IBS patients found significantly reduced TPH1 mRNA levels in both IBS subtypes[47].

CYTOKINES AND IBS

Cytokines are important mediators of inflammatory and immune responses. Cytokines have an established role in the inflammation of gastrointestinal tract, however the role in IBS is not yet clear. Genes involving various cytokines and inflammatory pathways have been studied. These involve genes for tumor necrosis factor-alpha (TNF-α), Transforming growth factor beta 1 (TGF-β1), interleukin-8 (IL-8) and interleukin-10 (IL-10). Production of these cytokines is under the genetic control and based on polymorphism involving genes coding these cytokines, high and low cytokine producing alleles been identified.

IL-10 gene polymorphism located at position 1082 constituting G to A substitution yields two types of alleles - allele G associated with high production of IL-10 whereas allele A associated with low production of IL-10[48]. Similarly for TNF-α, allele A and for TGF-β1, alleles T and G are identified as high producers[49]. Gonsalkorale *et al*[50], found significantly less number of high producer alleles of IL-10 in patients with IBS. Subsequently there were studies that highlighted the relationship between IBS and IL-10[51,52] but on the other hand others showed no association among the two[53]. Similarly the studies that explored the role of other cytokines like TNF-α also had conflicting results. Chang *et al*[52] did not find any positive association between IBS and TNF-α but van der Veek *et al*[53] on the other hand found that high producer genotypes of TNF-α were more prevalent in patients with IBS. Inconsistent findings of these and other studies led to a meta-analysis by Bashashati *et al*[54] that comprised of five studies with 529 IBS patients and looked at three cytokines - IL-10, TNF-α and TGF-β1. The meta-analysis found a decreased risk of IBS among high producer genotypes of IL-10 (1082, G/G), positive correlation between TNF (G/A genotype) and Asian IBS patients and failed to find any significant association between IBS and TGF-β1 gene polymorphism. Another more recent and larger meta-analysis by Bashashati *et al*[55] comprising of nine studies found no difference in blood IL-10 levels and IBS patients but based on gender stratification a significant association was found among men with IBS and lower IL-10 levels. With regards to TNF-α, the meta-analysis found that all IBS subtypes and women had significantly higher blood levels of TNF-α. In another meta-analysis comprising 8 studies with 928 IBS patients, all three known polymorphisms of IL-10 gene - rs1800870 (1082 G/A), rs1800871 (819C/T), and rs1800872 (592A/C) were explored. The meta-analysis concluded that one of the polymorphism was significantly associated with IBS in Caucasians (rs1800870), second (rs1800872) associated with IBS in Asians and third (rs1800871) had no association to IBS[56].

CANNABINOIDS

Δ9-Tetrahydrocannabinol, active ingredient of cannabis, acts through two cannabinoid receptors - CB1 and CB2. While CB1 mediates neurotransmitter release in the peripheral and central neuronal pathways, CB2 is associated with immune functions. CB1 receptors are present throughout the gastrointestinal tract

and endocannabinoids mediate their gastrointestinal effects through these receptors. Action of the endocannabinoids is terminated by their uptake and subsequent metabolism by the enzyme fatty acid amide hydrolase (FAAH)[57]. Gene coding for CB1 receptor has been mapped to chromosome 6 and polymorphism involving AAT triplet microsatellite flanking 3' end has been described in literature[58]. In a Korean study by Park *et al*[59] a significant association was found between IBS and the AAT triplet genotype with more than 10 repeats. Similar results were replicated by another Chinese study conducted by Jiang *et al*[60]. These authors also studied another single nucleotide polymorphism (rs324420) involving *FAAH* gene and found that A/A genotype was present less frequently in patients with IBS as compared to healthy controls but the difference was not statistically significant. In another study by Camilleri *et al*[61] comprising mainly of Caucasians investigated the association between phenotypic expression (colonic transit and rectal sensation) and genetic variations - AAT polymorphism as well as another polymorphism locus (rs806378) of CB1 receptor gene. The group found no association between AAT repeat polymorphism and phenotypic expression - colonic transit and rectal sensation. However, a significant association between D-IBS and colonic transit as well sensation rating of gas was found.

OTHER MEDIATORS

TNFSF15, also known as TL1a, is a member of tumor necrosis factor super family (TNFSF) of ligands. It binds to death receptor 3 (DR3) and mediates T cell proliferation, secretion of inflammatory cytokines and T cell immune responses. Polymorphism of TNFSF15 gene has been well established to be associated with inflammatory bowel disease. Its association with C-IBS was shown in a study by Zucchelli *et al*[62], whereas Swan *et al*[63] found it to be associated with D-IBS.

Cholecystokinin (CCK), a gastrointestinal tract peptide hormone acts through two receptors - CCK1 and CCK2. Genetic polymorphism of CCK1 was found more commonly among C-IBS and M-IBS patients in a study by Park *et al*[64]. In another study evaluating the effects of a CCK1 antagonist in C-IBS patients, gastric emptying was accelerated but no effect on overall colonic motility was found[65].

Enzyme catechol-o-methyltransferase (COMT) that degrades the catecholamines including adrenaline, noradrenaline and dopamine is another possible molecule related to IBS. Both, COMT gene polymorphism as well as IBS, have been shown to be associated with pain[66] and anxiety[67]. On these grounds the relationship between COMT gene polymorphism and IBS was also explored. Karling *et al* found that val/val allele of COMT gene encoding high COMT activity enzyme was more common among D-IBS patients. On the contrary, Chinese researchers Wang *et al*[68] found val/val allele to be protective against IBS, whereas the other allele met/met encoding reduced COMT activity enzyme significantly associated with elderly D-IBS patients. Interestingly in another study by Hall *et al*[69] investigating the placebo effect, the cases with allele (met/met) were found to be most responsive to placebos.

Single nucleotide polymorphism C825T on gene encoding β-3 subunit of the guanine nucleotide binding protein (GNβ3) was also explored. Studies from United States by Andresen *et al*[70] and Saito *et al*[71] found no relationship between this polymorphism and IBS patients. On the contrary, significant relationship among the GNB3 polymorphism and Korean as well Greek IBS patients was shown by Lee *et al*[72] and Markoutsaki *et al*[73] respectively. But other Korean study[74] as well as study from China did not show any association of the same polymorphism to IBS[68].

Hypothalamic hormone, corticotropin releasing hormone (CRH), which mediates its effects through two receptors - CRH1 and CRH2 has also been studied. Sato *et al*[75] explored three single nucleotide polymorphism of CRH1 gene and found significant association with IBS symptoms.

Mediators of autonomic system also have been implicated in pathogenesis of IBS. Association between autonomic dysregulation and IBS has been already shown in other studies[76,77]. α2-adrenergic receptors that are present on both presynaptic as well as postsynaptic locations and have three subtypes - 2A, 2B and 2C, are encoded by gene on chromosome 10. A 12 base pair deletion polymorphism of adrenergic receptor 2C (denoted α2C Del 322-325) and another single nucleotide polymorphism involving adrenergic receptor 2A gene (C1291G) were studied by Kim *et al*[78] Both the polymorphisms were found to have significantly higher odds (odds ratio 2.48 and 1.66 respectively) to be associated with C-IBS. In contrast, Sikander *et al*[79] in Indian IBS patients and Choi *et al*[80] in Korean IBS patients found an association between adrenergic receptor 2A gene polymorphism and D-IBS. Whereas in the study by Camilleri *et al*[81] no association was found between adrenergic receptor 2A gene polymorphism and IBS. Cholinergic muscarinic receptor type 3 gene polymorphism (rs3738435) was investigated by Onodera *et al*[82] Though no association was found between the polymorphism and different IBS subtypes, researchers found the polymorphism to be associated with the duration of disease.

So far the expedition to find a genetic basis for IBS has included wide array of studies ranging from epidemiological familial aggregation studies to gene polymorphism testing studies. Though these studies in several ways have shown the possible hereditary component of IBS but most of the studies have provided only weak evidence between the two. In the last decade gene search studies have focused more on detecting gene polymorphisms. Several possible gene polymorphisms have been shown to be associated

Table 2 Candidate genes associated with irritable bowel syndrome

Gene studied	Polymorphism
Serotonin or 5-HT reuptake transporter (SERT) gene – *SLC6A4*	5-HTT LPR – S and L alleles[23] rs25531 – A and G alleles[34] VNTR - alleles with 9, 10, 11 and 12 repeats[24]
5HT2A	1438 (G/A) and 102 (C/T)[41]
5HT3A	c.-42C>T[85]
5HT3E	rs62625044 - 76 (G/A)[44]
Tryptophan hydroxylase 1 (TPH1)	rs4537731 – 6526 (A/G)[45] rs684302 rs21105 rs1800532 – 218 (A/C) rs1799913 – 779 (A/C)
Tryptophan hydroxylase 2 (TPH2)	rs4570625 – 709 (G/T)[45]
Tumor necrosis factor–alpha (TNF-α)	308 (G/A)[53]
Transforming growth factor beta 1 (TGF-β1)	869 (T/C)[54] 915 (G/C)[54]
Interleukin-10 (IL-10)	rs1800870 - 1082 (G/A)[50] rs1800871 - 819 (C/T)[53] rs1800872 - 592 (A/C)[56]
Cannabinoid receptor 1 (CB1)	AAT triplets[59] rs324420[60] rs806378[61]
Tumor necrosis factor super family 15 (TNFSF15)	rs4263839[62]
Cholecystokinin 1(CCK1)	779 (T/C)[64]
Catechol-o-methyltransferase (COMT)	rs 4680 - 158 (Val/Met)[68]
Guanine nucleotide binding protein (GNβ3)	rs 5443 - 825 (C/T)[68]
Corticotropin releasing hormone receptor 1(CRH1)	rs110402[75] rs242924 rs7209436
α2-adrenergic receptor 2A	1291 (C/G)[80]
α2-adrenergic receptor 2C	Del 322-325[78]
Cholinergic muscarinic receptor type 3	rs3738435[82]

with IBS as a result of growing interest in gene polymorphisms. Even though the *P* values of these small studies show strength of association between the polymorphism tested and IBS but it cannot be taken as equivalent to genetic basis of IBS.

Genome-wide association studies

In addition to candidate gene-based association studies, association studies have also been conducted in a systematic, genome-wide manner. Genome wide association studies (GWAS) utilize high throughput genotyping techniques to assay hundreds of thousands of the most common form of genetic variation, the single-nucleotide polymorphism (SNP) and relate genotypes at these variants to the phenotypes of diseases and other traits[83]. This approach permits the interrogation of much of the common variation in the entire genome in thousands (or even hundreds of thousands) of unrelated individuals, achieving a higher positional resolution than is reasonably possible. In the first and so far the only GWAS by Weronica *et al* linkage was shown between IBS and two genes mapped to locus 7p22.1. The two genes mapped to this locus were KDEL receptor 2 gene (*KDELR2*) and glutamate receptor ionotropic delta 2 interacting protein (GRID2IP)[84,85].

FUTURE DIRECTIONS

The field of genetics exploring the underlying potential IBS genes is rapidly evolving. However, huge gaps in our understanding of this complex disorder remain and need further study. Genetic research efforts that are focused on studying the candidate gene polymorphism have so far provided the weak evidence at the best. Gene linkage studies and genome wide association studies are needed to understand the deficiencies in our current knowledge. In the future studies, it will be also of importance to explore how the environment influences these newly discovered genes.

IBS is a complex functional gastrointestinal disorder that poses a great challenge to both, the affected patients and the physicians treating the ones affected. Historically, underlying pathophysiology is believed to be interplay of hypersensitive gut with key contributions from psychological, social and environmental factors. Genetic basis has also been suggested by familial aggregation of cases as well as high concordance rate among monozygotic twins than dizygotic twins. Gene hunt efforts, so far, have explored several potential candidate genes (Table 2) and shown association between polymorphic gene loci and different IBS phenotypes. While studies so far have been less than emphatic in their results due to small sample sizes, varying criteria used to diagnose IBS, heterogeneity of methods used and ethnic differences of the participants tested, there is convincing evidence that a proportion of IBS are due to additive genetic effects. As an alternative to current gene specific candidate driven approach, larger and non-candidate gene driven studies in the form of GWAS are needed to understand this common and complex disorder. Such genome wide studies may provide much needed insight into the genetic susceptibility of IBS and could aid in development of novel therapeutic strategies to diagnose, treat and prevent this disorder.

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TOPIC HIGHLIGHT

2015 Advances in Irritable Bowel Syndrome

Irritable bowel syndrome and chronic constipation: Fact and fiction

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Abstract

Irritable bowel syndrome (IBS) and functional constipation (FC) are the most common functional gastrointestinal disorders. According to the Rome Ⅲ Criteria these two disorders should be theoretically separated mainly by the presence of abdominal pain or discomfort relieved by defecation (typical of IBS) and they should be mutually exclusive. However, many gastroenterologists have serious doubts as regards a clear separation. Both IBS-C and FC, often associated with many other functional digestive and non digestive disorders, are responsible for a low quality of life. The impact of the media on patients' perception of these topics is sometimes disruptive, often suggesting a distorted view of pathophysiology, diagnosis and therapy. These messages frequently overlap with previous subjective opinions and are further processed on the basis of the different culture and the previous experience of the constipated patients, often producing odd, useless or even dangerous behaviors. The aim of this review was to analyze the most common patients' beliefs about IBS-C and CC, helping physicians to understand where they should focus their attention when communicating with patients, detecting false opinions and misconceptions and correcting them on the basis of scientific evidence.

Key words: Irritable bowel syndrome; Chronic constipation; Functional constipation

Core tip: The media often suggests a distorted view of pathophysiology, diagnosis and therapy of irritable bowel syndrome and chronic constipation. These messages frequently overlap with previous subjective opinions and are further processed on the basis of the different culture and the previous experience of the constipated patients, often producing odd, useless or even dangerous behaviors. The aim of this review was to analyze the most common patients' beliefs regarding these disorders, helping physicians to understand where they should focus their attention when communicating with patients, detecting false opinions and misconceptions and correcting them on the basis of scientific evidence.

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STATE OF THE ART

Irritable bowel syndrome (IBS) and functional constipation (FC) are the most common functional gastrointestinal disorders. They negatively affect quality of life and are associated with a significant economic burden related to direct and indirect annual healthcare costs[1,2].

Most gastroenterologists, mainly on a scientific basis, use the Rome Ⅲ Criteria[3] (Figure 1), which divide non organic constipation into functional constipation (FC) and IBS-C. These two different categories are theoretically separated mainly by the presence of abdominal pain or discomfort relieved by defecation (typical of IBS) and they should be mutually exclusive: in FC abdominal pain and discomfort are not included in the definition. In clinical practice the situation is somewhat different and many gastroenterologists have serious doubts about clearly separating these two disorders[4-7].

Population-based studies carried out in North America reveal that between 1.9% and 27.2% of individuals experience constipation, with most estimates ranging from 12% to 19%[8]. These different values are probably due to the fact that constipation is a symptom rather than a disease, susceptible to different and subjective interpretations of a real or imagined disturbance of bowel function[9]. This generates many different definitions, some focusing on the interval between defecations (number of weekly defecations), and others reflecting the sensation of difficult defecation or incomplete bowel movements, with an objective assessment of stool consistency being rarely used in clinical practice[10]. A recent meta-analysis shows an IBS prevalence of 11.2%. The prevalence varied according to the countries and criteria used to define IBS. The lowest prevalence appeared in South Asia (7.0%) and the highest in South America (21%). Women are at a slightly higher risk of both CC and IBS than men[11].

Both IBS-C and CC are often associated with functional digestive and non digestive disorders[12-16]. In particular, IBS-C patients show a higher prevalence of psychological disorder, a higher rate of depression and anxiety and a lower quality of life than patients with IBS-D[17].

The impact of the media on the perception of these topics is often disruptive because it proposes a distorted view of these disorders. This message frequently overlaps with previous subjective opinions and is further processed on the basis of the different culture of our patients, often producing odd, unhelpful or even dangerous behaviors. The aims of this review were to analyze the false beliefs of IBS-C and CC patients, which are influenced by a wide variety of factors, both internal to the patients themselves as well as the external media messages. Moreover, we will lay out a framework to help us understand where the clinicians should focus the patients' attention when communicating with them.

I underwent a barium enema/colonoscopy and they found my colon longer than normal; they told me it's the reason why I'm constipated and it would be better to shorten it. Could we cut a good piece away?

For the first time, Sir Arbuthnot Lane in the early 1900s formulated a theory according to which colonic kinking and/or an excessively long colon could cause fecal stasis, favoring intestinal absorption of water and toxins and leading to a systemic dysfunction. Consequently, colon bypass or total colectomy were suggested for indications ranging from lassitude to epilepsy[18]. By the 1920s, surgical treatment of intestinal stasis had progressively fallen into disfavor. In fact, there is very little evidence to support either procedures aimed at shortening the colon of patients which is elongated but not dilated (dolichocolon), or indeed any surgical operations designed to straighten colonic kinks or intestinal loops, except in the presence of a volvulus causing an acute bowel obstruction). Actually no study has correlated colon length with colonic transit[19].

Nowadays, if a surgical approach to the colon is suggested, it is a total colectomy. This is reserved for only very few cases. These are the most severe and refractory cases of constipation, in which imaging techniques and manometric findings have shown the presence of colonic inertia, usually linked to neuropathy and/or myopathy involving the whole colon[20]. In these cases a very careful preoperative evaluation is mandatory to verify the real indication for colectomy, as is confirmed by a recent Italian study[21]: 450

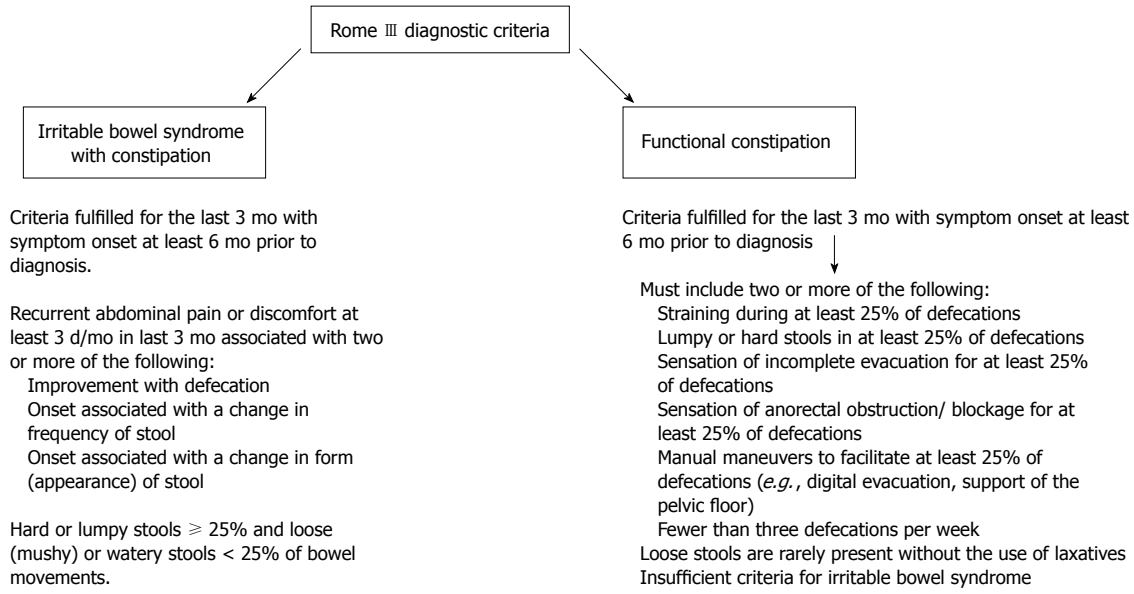

Figure 1 Rome Ⅲ criteria for irritable bowel syndrome and functional constipation.

patients with chronic constipation were evaluated and 33 patients with a diagnosis of slow-transit constipation that had not improved with medical or rehabilitative treatment - after a meticulous assessment of colonic motility, they underwent colectomy. All patients except one had a positive outcome of the colectomy, thus improving their quality of life.

I do not eat citrus fruits because they cause constipation

It is a common belief that citrus fruits have constipating effects and could display a positive action in diarrheic syndromes. On the contrary, they are rich in pectin, which is an indigestible carbohydrate commonly contained in the cellular wall of the vegetal tissues. It is a soluble fiber representing a not negligible amount (0.5%-3.5%) of the fresh weight of the citrus fruits and particularly it represents about 30% of the fresh weight of the peel of citrus fruits[22]. For more than fifty years[23] pectin has been shown to be an adjuvant in controlling constipation. So actually citrus fruits can be useful in the treatment of constipation and in absolutely no way do they worsen it.

Furthermore, citrus fruits, like in general other plant products, contain water, sorbitol, fructose, fiber, and phytochemicals. The only fruit that is not recommended for a constipated patient is the banana, especially if unripe: an unripe banana contains 100-250 mg tannins/100 g and has a high amylase-resistant starch content (tannin acid reduces small intestinal secretions and inhibits peristalsis). This fruit can therefore have a constipating effect[24].

My neighbor is constipated like me. She takes a small pill at bed time and everything is fine! Why doesn't the same happen to me?

Many people think that constipation is a single disease. Actually it is a complex multifaceted syndrome involving many different causes (Table 1). Moreover, the term "primary constipation" itself hides different conditions, such as irritable bowel syndrome with constipation (IBS-C), functional constipation, functional defecation disorders and rectal hyposensitivity (Figure 1).

IBS-C, based on the Rome Criteria[3], is defined as a recurrent abdominal pain or discomfort at least 3 d per month in the last 3 mo associated with 2 or more of the following: (1) improvement with defecation; (2) onset associated with a change in frequency of stool; and (3) onset associated with a change in form (appearance) of stool. The stools are hard or lumpy (Bristol Stool Form 1-2) ⩾ 25% and loose (mushy) or watery (Bristol Stool Form 6-7) ⩽ 25% of bowel movements, in the absence of use of antidiarrheals or laxatives.

Functional constipation, on the other hand, is a functional bowel disorder that presents as persistently difficult, infrequent, or seemingly incomplete defecation, which do not meet IBS criteria. Usually, there is no demonstrable physiological abnormality.

Functional defecation disorders are characterized by paradoxical contraction or inadequate relaxation of the pelvic floor muscles during attempted defecation (dyssynergic defecation) or inadequate propulsive forces during attempted defecation (inadequate defecatory propulsion)[25] (Table 2).

Rectal hyposensitivity is a relatively new disorder defined by Gladman *et al*[26] as an elevation beyond the normal range in the perception of at least one of the sensory threshold volumes during anorectal manometry. In a population of 1351 patients with anorectal symptoms, rectal hyposensitivity was present in 23% of patients with constipation, 10% of patients with fecal incontinence and in 27% of patients with incontinence associated with constipation.

Table 1 Secondary causes of constipation

Drugs	Opiates, anticholinergics, antidepressants, anticonvulsants, calcium channel blockers
Endocrine and metabolic diseases	Hypothyroidism, hypercalcemia, diabetes, porphyria
Neurological diseases	Parkinson's disease, multiple sclerosis, spinal cord injury, autonomic neuropathy
Psychiatric disorders	Depression, eating disorders, obsessive disorders
Gastrointestinal diseases	Bowel obstruction, agangliosi, myopathies, neuropathies, megacolon/megarectum, anal atresia, anal stenosis

Therefore, rectal hyposensitivity can be considered as an important cause of constipation.

There are as yet no specific criteria that can differentiate the subtypes of chronic constipation based on anamnesis[25]. Also performing a full assessment of defecation using specific tests (*e.g.*, anorectal manometry, colonic transit time and defecography) may not be enough to distinguish these different conditions[4-6]. However, a careful attempt to understand the pathophysiological mechanisms underlying the constipation of each patient is mandatory in order to suggest an effective therapy. This should be strictly tailored to each individual patient and therefore different from one patient to another. Therefore, it is not at all unusual that a drug effective in one patient does not work in another.

Bowel frequency bothers me: I don't have a bowel movement every day!

The frequency of bowel movements is usually considered a "key point" for the diagnosis of chronic constipation and IBS-C, but it is neither a sufficient nor a necessary issue[3]. A decrease in bowel frequency usually prompts the patient to define her/himself as suffering from constipation, However, according the Rome Ⅲ criteria, the item "fewer than three defecations per week" must be accompanied by at least one of the other five characteristics in at least 25% of the defecations[3] (Figure 1).

In clinical practice it is mandatory to explain to the patients that most normal people have a bowel frequency ranging from 3 times a day to 3 times a week and that it is not necessary to have a bowel movement every day.

The term "constipation" often has different meanings for patients and physicians[6,27] and it is necessary to understand what exactly patients mean with "bowel movements". Sometimes both patients and physicians consider bowel movements simply as attempts at defecation or the defecation of small, unsatisfying, pieces of feces which oblige patients to go to the toilet many times a day. It is a gross mistake to consider this to be an increase in bowel frequency, possibly indicating a diarrhea. This is simply "fragmented defecation", typical of many constipated patients, especially of those affected with obstructed defecation. Moreover, some patients do not consider "bowel movement" when they use enemas or suppositories and this can cause a misunderstanding with their physician. It is important to investigate all attempts to defecate so as to better understand what exactly patients mean.

I'm too constipated: I'll get colon cancer, sooner or later!

Prospective cross-sectional surveys and more recent meta-analysis demonstrate no increase in the prevalence of colorectal cancer in patients or individuals with constipation. The significant association observed in case-control studies may be related to a combination of poor study quality and recall bias among enrolled patients. When patients undergo colonoscopy for constipation as a main indication of the procedure the diagnosis of colorectal cancer is less common than in patients undergoing colonoscopy for other gastrointestinal symptoms. Therefore, the use of lower GI investigations to exclude colon cancer in patients presenting with constipation, in the absence of other "red flags", should be discouraged[28-30].

If I haven't got my bowel movement every day I feel like a wet rag!

Constipated patients often refer fears linked to an undefined and unspecified concept of poisoning. They often imagine that fecal stasis has systemic effects and a range of intestinal and extra intestinal manifestations such as feelings of a bitter taste in the mouth, dyspepsia, headache and tiredness, etc. This is likely related simply to a delayed intestinal transit time[19].

There is no scientific evidence to support the idea that there are diseases related to fecal stasis associated with the mechanisms of self-intoxication. The lack of specific symptoms and the improvement related to the act of defecation (more typical for mechanical effects and not for systemic effects) has led the scientific community to abandon the hypothesis of such relationships[18].

The presence of comorbidities associated with both constipation and IBS-C (psychiatric disorders, fibromyalgia, headache, sleep disturbance, dyspareunia, recurrent urinary infections) have been confirmed by many studies[16,31]. Psychiatric and extra intestinal comorbidity impacts on the quality of life and bowel symptom burden in functional GI disorders[32].

The relationship with the brain-gut axis is not completely understood. Probably, the dysbiosis caused by constipation induces slight inflammation, due to altered barrier dysfunction, and a release of pro-inflammatory cytokines, with consequent dysregulation

of the brain-gut and gut-brain axes[33-35].

My stools are like a sausage, a Havana cigar, a snake, fettuccini, a tubular tire, small black olives…

Fecal consistency is often overlooked in the anamnesis, both because of patient's embarrassment and because of the variability and the subjectivity of their descriptions (a great many bizarre and surprising metaphors are frequently heard in our surgeries…)[27]. However, fecal consistency is an important clue to the transit time and must be clarified both in the clinical practice of prescribing a tailored and efficacious therapy and also within a clinical research setting. With this purpose in mind the Bristol scale offers a valuable aid in order to avoid errors related to excessively vague and subjective descriptions or to patient's recall bias, and its use should be encouraged in everyday practice[36,37].

Doctor, do we really need to do a rectal examination? I' m a bit embarrassed to have rectal examination…!

Digital rectal examination (DRE) is the most commonly used method to clinically assess anal tone although there is a lack of consensus in the current literature with regards to its accuracy and reliability. A comparison of DRE findings by an experienced gastroenterologist alongside objective ano-rectal manometry to establish anal tone reported DRE to be of low specificity and sensitivity and too inaccurate to use as a clinical finding[38]. Many patients consider DRE bothersome or are afraid of feeling pain or more often than not are ashamed of undergoing this kind of medical examination. However, DRE is strongly recommended to verify if there is any fecal impaction in the rectum and to detect any early form of rectal cancer[39] or benign diseases such as polyps, hemorrhoids (internal), anal fistula (low or high), rectal prolapse, *etc*.[40,41]. Recent data have shown that DRE is performed in only 56% of Italian gastroenterological consultations[16].

Doctor, you suggested a lot of annoying tests: aren't there too many of them? Are they really necessary for my constipation?

Gut functional disorders should be diagnosed using the Rome criteria[3], but in clinical practice they are frequently dealt with by means of an exclusion criteria approach that takes into account the exclusion of organic diseases (alarm signs)[42].

The presence of alarm features alerts the clinician to the possibility of an organic, rather than a functional disease process, and usually signals the need for testing in order to rule out an underlying organic disorder. These alarm features include rectal bleeding, weight loss, iron- deficiency anemia, nocturnal symptoms, and a family history of certain organic diseases such as colorectal cancer and inflammatory bowel disease[43]. Their presence may indicate the need for colonoscopy, colon-CT, or barium enema to exclude the presence of organic lesion or an associated disease[20,43-45].

In patients without alarm symptoms, a "step-by-step" diagnostic approach is suggested, even if it frequently overlaps with life-style changes or a pharmacological treatment to resolve symptoms.

In patients with unsolved, recurrent signs and symptoms haematological, faecal and radiologic or instrumental approaches are considered necessary and advised[20,43,44].

Integrated information coming from anorectal (sometimes gastrojejunal and/or colonic) manometry, Rx defecography and colonic transit time are often necessary because, up to now, no test has resulted in being completely exhaustive in assessing the pathophysiological mechanisms of defecation. Indeed, none of them studies defecation in normal conditions, but uses catheters, probes, different contrast media or simply in a position (usually left lateral position) not normally used in daily normal defecation. Last but not least, it should be considered that patients undergoing these tests are generally requested to simulate defecation, which is such an intimate and private act, in front of doctors, nurses and technicians. This can generate obvious embarrassment, hindering proper conduct and reducing the reliability of a single test: using more than one test can reduce errors and improve the quality of the assessment of the defecatory pattern[20,44].

In Figure 2 we report a diagnostic flow-chart approach to chronic constipation.

Could physical activity help me to have more satisfying defecation?

It is well known that people who undertake more physical activity have a lower prevalence and a better control of constipation. There is some evidence that bowel function can correlate to physical activity, but other factors may very well be involved. For instance, in the elderly many cofactors such as diet, medications, cognitive and psychological condition are likely to play a role and physical activity is only a part of a multifaceted and multidisciplinary therapeutic approach. On the other hand, in the young severely constipated patients physical activity probably does not improve bowel function[19,43].

My bowel function and abdominal pain are worse after eating food! I usually drink a lot of water but I don't feel better. I eat a lot of fibers and drink plenty of liters of water every day to improve my bowel function but I'm quite unsatisfied.

Patients with IBS-C and CC commonly believe that specific dietary products contribute to their symptoms while other foods could prevent the same disturbances. Most patients increase the use of dietary fiber to regularize bowel function and to reduce meteorism

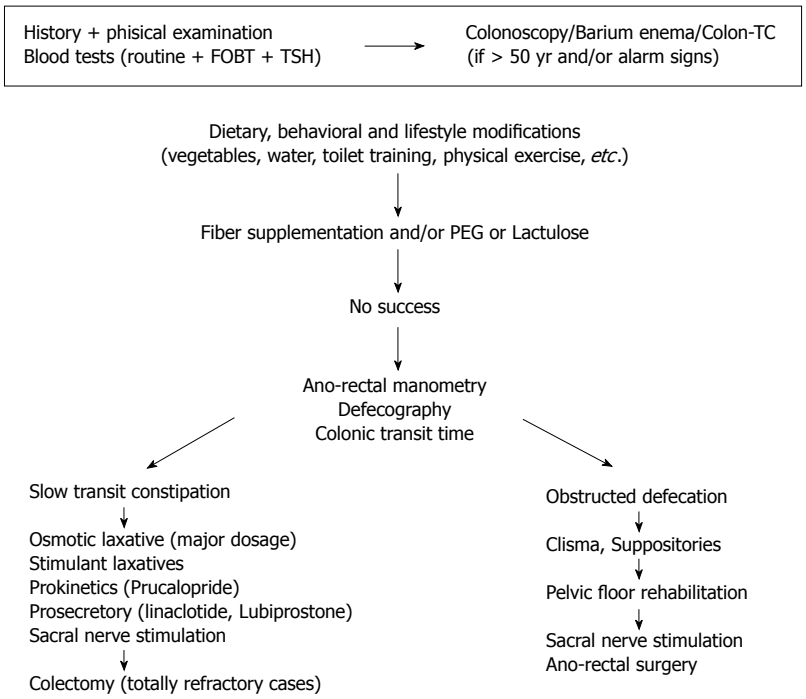

Figure 2 Multi-step management of chronic constipation. After a careful history and some blood tests, if there are no alarm signs and the patient is < 50 years old the first line approach encompasses a correction of lifestyle and dietary habits (on the contrary a colonic morphological assessment by using colonoscopy or barium enema or colon-CT is advisable). If the results are not satisfying, fiber supplementation and/or therapy with polyethylene glycol (PEG) or lactulose are advisable. Should there be unsatisfactory results, "second line" drug therapy, using saline or stimulant or softening laxatives, could be suitable and, possibly using or adding the new drugs with a prokinetic or prosecretory effect (prucalopride, lubiprostone, linaclotide). At this step performing some diagnostic "second level" exams (*e.g.*, anorectal manometry and/or defecography and/or colonic transit time) should be taken into account. Pelvic floor rehabilitation and subsequently anorectal surgery or sacral nerve stimulation should be considered on the basis of the results of the "second level" exams. Colectomy represents the "last resort" and should be suggested only for patients with "inertia coli" only after performing also colonic and gastrojejunal manometry. FOBT: Fecal occult blood test.

and pain. There is some evidence that patients taking soluble fiber (psyllium, guar) have significant symptom relief, whereas insoluble fiber (bran) shows no clinical benefit and actually may worsen symptoms in many cases[45]. The impairment of symptoms after an increase in dietetic fiber can also be due to the kind of constipation. It is well known that patients with dyssynergic defecation or with severe slow transit constipation can meet more severe symptoms after heavy fiber supplementation because it can further slow down colonic transit or it simply increases the amount of feces in the rectum, which the patient is not able to empty[46]. Another common conviction is that constipation can be improved by drinking a considerable amount of water. Many patients force themselves to drink more than 2 L of water a day. Data currently available do not suggest that stool consistency and frequency of evacuation can be significantly modified by increasing fluid ingestion by more than 2 L a day[47].

Furthermore, patients often associate their complaints with the ingestion of foods containing fructans, galactans, lactose, fructose, sorbitol, xylitol, and mannitol (fermentable oligo-di-monosaccharides and polyols; FODMAPs), which mainly increase abdominal bloating and distension[48]. Patients with IBS, but without celiac disease, may reach satisfactory symptom control with a gluten-free diet[49]. Because of conflicting evidence regarding dietary implications in functional disorders, only a double-blind food-specific challenge will discriminate between a true and a false food-sensitivity in IBS patients.

I sometimes take a lot of PEG but I don't get good results. Why does my cousin have good defecation after drinking a coffee and eating a kiwi at breakfast?

Patient skepticism characterizes the relationship with medical professionals. Patients with IBS-C and functional chronic constipation (CC) are often not satisfied with the treatment they are receiving and actively, and sometimes compulsively, seek information on possible alternatives.

The main source of information to obtain knowledge and to know how to deal with the disease is the general practitioner, the pharmacist, but also friends and relatives and the mass media (magazine, TV, the Internet, *etc.*). The gastroenterologist is often at the end of the queue of this informative process.

Laxatives are the most widely used medications to improve bowel function both in IBS-C and CC. In particular, polyethylene glycol (PEG) is more effective than lactulose and stimulant agents in increasing stool frequency and improving stool consistency, and it is considered the first choice of treatment for CC[20]. It should be taken on a regular basis, using it as an intestinal regulator and not to induce bowel

Table 2 Rome Ⅲ diagnostic criteria for functional defecation disorders

Criteria fulfilled for the last 3 mo with symptom onset at least 6 mo prior to diagnosis
The patient must satisfy diagnostic criteria for functional constipation
During repeated attempts to defecate must have at least two of the following:
Evidence of impaired evacuation, based on balloon expulsion test or imaging
Inappropriate contraction of the pelvic floor muscles (*e.g.*, anal sphincter or puborectalis) or less than 20% relaxation of basal resting sphincter pressure by manometry, imaging, or EMG
Inadequate propulsive forces assessed by manometry or imaging

movement, as a stimulant laxative. The starting dose, even a low dose, has to be progressively adjusted by the patient who has to find her/his own effective dosage. Generally speaking, the success of the constipation therapy is variable, depending on patients' age and compliance, duration of disease, impairment of colonic transit and/or pelvic floor, comorbidities and psychological disturbances. In the presence of only a partial clinical success or failure of traditional therapy, a second line treatment (prucalopride in CC and linaclotide in IBS-C and lubiprostone both in IBS-C and CC) is available today[44-50]. In more severe cases of IBS-C and CC higher drug dosages than normal and/or combined therapies with laxatives with different mechanisms of action (*e.g.*, osmotic plus stimulant drugs or osmotic plus prokinetic drugs or prokinetic plus secretagogue drugs) may be necessary.

I couldn't live without laxatives: I have become dependent on laxatives!

Some IBS/CC patients are "dependent" on laxatives to constantly maintain bowel movements without complaints. In most cases this addiction does not have a pharmacological basis, because most laxatives are not absorbed and none cross the blood brain barrier, but it is based only on psychological and behavioral factors[51]. However, some patients, usually with psychiatric or psychological problems, abuse the use of laxatives for extended time periods. After stopping laxatives a "rebound constipation" does not seem to represent a real, frequent problem[19].

Premature pharmacological tolerance can develop if there is prolonged treatment and this can represent another worry for constipated patients. Tolerance to laxatives has not been systematically studied in humans. However, tolerance (in particular to stimulant products) seems to occur in the most severely constipated patients for whom other products are ineffective. On the other hand, tolerance seems to be uncommon in the majority of cases[19,51].

Can laxatives cause risks for my health?

Patients are obviously interested in the potential side effects of these products. Laxatives can determine electrolyte disturbances or abdominal complaints such as abdominal pain and intestinal bloating. However, this can be minimized with an appropriate selection of the drug (osmotic laxatives are better than stimulant agents) and the tailored dose for a given patient. In particular, stimulant laxatives are preferred due to their rapid action even if, due to their mechanism of action, they tend to induce abdominal pain more frequently than osmotic laxatives[19]. A typical fear of patients regards the risk of developing colorectal cancer after a chronic use of laxatives. Particularly pseudomelanosis coli, merely a pigmentation of the colon surface due to the accumulation of lipofuscin in macrophages as a consequence of the chronic use of anthraquinones, was considered in the past to be an expression of mucosal damage, should not in any way be considered as related to colon cancer[52,53]. In general, constipation does not appear to be associated with an increased risk of cancer and there are no solid data supporting the idea that stimulant agents are a specific risk factor for colon cancer[19].

A friend advised me to take herbal medications. What about the efficacy and safety of these products?

Another frequently asked question regards the use of herbal medication. Some IBS/CC patients consider this approach safer than traditional therapy. Herbal medications have been used in many countries for many centuries for the treatment of patients with constipation. Many patients underestimate the possible metabolic interaction of herbal products compared to the drugs they currently take, mistakedly presuming that all that is natural is beneficial, or at least harmless[54]. Moreover, many of these "natural products" are bought by the patients without any medical prescription or supervision and without any information regarding their composition and origin. Indeed, there is a lack of controlled data supporting the safety and the efficacy of these treatments and no conclusive data are available regarding the possible toxicity of any herbal mixtures[55,56].

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TOPIC HIGHLIGHT

2015 Advances in Irritable Bowel Syndrome

Role of environmental pollution in irritable bowel syndrome

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Abstract

Irritable bowel syndrome (IBS), with the prevalence of 10%-20 % of the population has become an emerging problem worldwide. IBS is a functional gastrointestinal (GI) disorder characterized by abdominal pain or discomfort and altered bowel habits. The etiology of IBS contains genetic, psychological, and immunological factors, and has not been fully elucidated; of note, recent studies also point at environmental pollution and its role in the development of functional GI diseases. In this review we focus on several environmental factors, such as bacterial contamination, air pollution, radiation and even stress as potential triggers of IBS. We discuss associated disturbances in homeostasis, such as changes in intestinal microbiome and related pathophysiological mechanisms. Based on the effect of environmental factors on the GI tract, we also propose novel targets in IBS treatment.

Key words: Irritable bowel syndrome; Environmental pollution; Air pollution; Stress; Post infectious irritable bowel syndrome

Core tip: Irritable bowel syndrome (IBS) is a functional gastrointestinal disorder characterized by abdominal pain or discomfort and altered bowel habits. The etiology of IBS has not been fully elucidated; however, recent studies point at environmental pollution and its role in the development of IBS. Here we focus on several environmental factors, such as bacterial contamination, air pollution, radiation and even stress as potential triggers of IBS; we also propose novel targets in IBS treatment.

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INTRODUCTION

Irritable bowel syndrome (IBS) has become a critical issue worldwide, with the prevalence of 10%-20% of the population[1]. IBS is a heterogenous functional disorder, characterized by abdominal pain or discomfort and altered bowel habits. While there is no reliable biomarker, Rome Ⅲ diagnostic criteria define IBS as a recurrent pain or discomfort for at least 3 d per month in the past 3 mo. In addition, the symptoms have to be associated with the two or more of the following: relief by defecation and the onset associated with the change of the frequency or form of the stool. IBS can be classified into four subtypes according to stool form, namely IBS with constipation (IBS-C), IBS with diarrhea (IBS-D), mixed IBS (IBS-M) and unsubtyped IBS (IBS-U)[2]. Although not life-threatening, IBS has a negative economic effect on health service, among others due to the fact that the patient may need to undergo expensive tests and treatments before proper diagnosis. According to Maxion-Bergemann *et al*[3] total direct cost estimates per IBS patient range from USD 348-8750 per year. Moreover, IBS is an important reason for patients' absenteeism from work; the average number of days off per year due to IBS was between 8.5 to 21.6. Finally, IBS provokes multiple comorbidities, such as chronic fatigue, headache, insomnia, psychiatric disturbances, dyspepsia and gastro-esophageal reflux which, taken together with direct symptoms of IBS lead to impaired quality of life[4].

The etiology of IBS is undoubtedly multi-factorial, but its understanding is unsatisfactory due to lack of evident pathological abnormalities and infallible biomarkers. Currently, IBS is viewed as a disorder to which genetic, immune, and psychological factors, as well as alterations in microbiota, visceral perception and gastrointestinal (GI) motility contribute; diet and changes in brain-gut axis activity may also count (Figure 1). It still remains unclear which of these factors is the main trigger for the onset of IBS.

Recently, the world has been seeing a dramatic rise in population growth in urban areas. As urban populations grow, the quality of the environment, and especially urban air pollution, will play an increasingly important role in public health. Consequently, the disease burden due to air pollution will be on the rise. While research on airborne pollutants has drawn attention mostly to respiratory and cardiovascular systems[5], emerging evidence suggests that these pollutants may also have adverse effects on the GI tract, being involved in the pathophysiology of inflammatory bowel disease (IBD)[6], appendicitis[7,8], and possibly irritable bowel syndrome[9]. While for instance smoking may affect the disease onset in IBD[10], the role of environmental pollution in IBS has not been fully elucidated.

In this review, we focus on the impact of environmental pollution - in its broadest sense - on development of IBS. We refer to current knowledge on the prevalence of IBS in regions with higher environmental pollution rate and discuss potential association between pollution and development of the disease.

MICROBIOLOGICAL POLLUTION

Postinfectious IBS

Postinfectious IBS (PI-IBS) is a particular case of IBS, which is caused by acute infectious gastroenteritis; in fact, it is considered as the most common cause of IBS[11]. It was shown in prospective studies that 4% to 36% patients suffer from PI-IBS because of previous infection[12]. Noteworthy, the first reports on the disease date back already to 50 years ago[13]. The pathogens that contribute to PI-IBS are *Campylobacter jejuni*, *Salmonella enterica*, *Shigella sonnei*, *Escherichia coli* O157:H7, noroviruses and *Giardia lamblia*. The disease symptoms are not immediate, it takes approximately 8-10 years to develop a full-blown PI-IBS[14]. The duration of infection is crucial; for example, a fortnight-long *Shigella sonnei* infection was considerably more associated with PI-IBS than a week-long one (RR = 4.6)[15].

The most common alterations during PI-IBS are found in mucosal cells. Contrary to healthy volunteers and control patients, in which *Campylobacter jejuni* infection had no consequences, rectal mucosal enterochromaffin cell (EC) levels are increased in PI-IBS patients[16]. Moreover, mucosal barrier is mutilated by *Campylobacter jejuni* infection, thus the transepithelial electrical resistance is decreased[17]. Of note, changes in mucosal barrier function are considered to be associated with the tumor necrosis factor α (TNF-α) pathway[18]. On the other hand, when post-Shigella IBS is concerned, rise in ileal mast cells and in nerve fibers immunoreactive for neuron-specific enolase, substance P, and 5-hydroxytryptamine can be observed[19]. Finally, postviral PI-IBS has also been described. However, its pathophysiology remains unclear; of note, it has been suggested that IBS after norovirus infection is rather temporary, as compared to post-bacterial[20,21].

Walkerton crisis as an example of bacterial pollution

In May 2000, an ecological crisis because of procedural mistakes took place in Walkerton, Ontario, Canada. *Escherichia coli* O157:H7 and *Campylobacter jejuni* entered the drinking water system, while chlorine level (which was not monitored regularly) was too low to counteract the pollution[22]. This event caused 7 fatalities and over 2300 victims among 4800 Walkerton residents. A cohort study conducted by

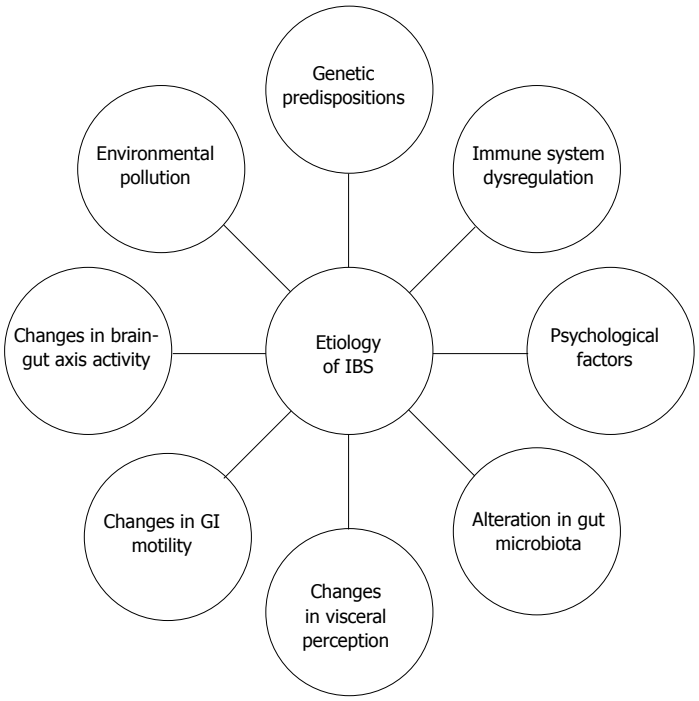

Figure 1 Multifactorial etiology of irritable bowel syndrome. IBS: Irritable bowel syndrome; GI: Gastrointestinal.

Marshall *et al*[23] showed that the prevalence of PI-IBS in the local population increased significantly in comparison to healthy controls. Interestingly, 8 years after the incidence, the prevalence decreased from 28.3% (after about 3 years) to 15.4%; however, it was still higher than in unaffected subjects (OR = 3.12; 95%CI: 1.99-5.04).

Acquiring the cohort of 2069 adults after Walkerton outbreak was a great opportunity to investigate the long-term outcomes of water contamination on the GI tract function and more studies are expected. Moreover, it clearly showed that bacterial pollution resulting from human error, which is plausible, may have an effect that will be observed for several years after it occurred.

AIR POLLUTION

Air pollution is a mixture of a number of substances including gases, such as carbon dioxide, ozone, nitric oxide, volatile organic compounds (benzene) and particulate matter (PM), with the latter being the one mostly responsible for adverse health conditions. Of note, daily ingestion of PM on a typical Western diet is estimated at 10^{12}-10^{14} particles per individual[24,25]. The GI tract is highly susceptible to PM (as well as smoking) and exposure to air pollution may exacerbate systemic inflammation or lead to oxidative damage of colonic mucosa[10]. For example, a study performed by Dybdahl *et al*[26] indicated that the exposure to diesel exhaust particles elaborated DNA adducts and oxidative stress, resulting in DNA strand breaks, apoptosis and protein oxidation in colon mucosa. Another study demonstrated that mice deficient in apolipoprotein E -/- exposed to low concentration of $PM_{2.5}$ developed systemic inflammation, which was expressed mainly by vascular inflammation and increased atherosclerosis[27].

Air pollution and gut microbiome

Despite the well-studied effects of environmental pollutants on several health conditions[28,29], little is known on how air pollution impacts the gut microbiome. Kish *et al*[30] showed that pollutant particles ingested with chow altered gut microbiota composition by significant changes in the relative amounts of *Bacteroidetes*, *Firmicutes* and *Verrucomicrobia*. Moreover, mice exposed to polychlorinated biphenyls from contaminated food had decreased levels of *Proteobacteria* and increased levels of *Bacteroidetes*[31]. These results suggest that environmental pollutants may alter significantly the microbiome composition.

Air pollution and IBS-related pain

Non-specific abdominal pain is one of the crucial symptoms of IBS. In the years 1992-2002 and 1997-2002, Kaplan *et al*[9] performed two studies (in Edmonton and Montreal, respectively) focusing on the possible association between nonspecific abdominal pain and air pollution. The report published in 2012 showed that the young individuals aged 15 to 24 years, with preponderance of women, had the highest prevalence of non-specific abdominal pain and were significantly more likely to visit emergency department when the indicators of air pollution, such as CO, particles < 2.5 ($PM_{2.5}$) μm, SO_2, and NO_2 were elevated[9]. Until today, the mechanism by which the air pollutants exacerbate abdominal pain has not been clarified. It has been suggested that increased IL-8 secretion from small bowel and changes in composition of colonic microflora[32] or alterations in colonic motility[9] may be the key factors. Moreover,

in the same study it has been demonstrated that the exposure of mice to air pollutant EHC-6802 particles, recovered from filters of the single-pass air purification led to increased pain response. It is likely that air pollution may exacerbate systemic inflammation[27] and cause oxidative damage of colonic mucosa[26], what contributes to the occurrence of IBS symptoms.

RADIOACTIVE POLLUTION

Nuclear energy is a potent source of electric power in contemporary world. As the statistics show, it contributed to 10.8% of total world production of electricity in 2013[33]. On the one hand nuclear power is considered to be less harmful to the natural environment than conventional sources of energy since it does not produce common pollutants, such as greenhouse gases. On the other hand the humanity witnessed several nuclear reactor accidents in the past few years, which contaminated surrounding areas with radiation for several years. One of the most damaging was the explosion of nuclear power plant in Chernobyl (currently Ukraine) in 1986. As a result, about 14 EBq of radioactive substances were released to the atmosphere, contaminating the area of more than 200000 km^2 in Europe. Up to 71% of radioactive caesium (^{137}Cs), which affects inhabited areas to this day ($t_{1/2}$ = 30.17 y), deposited in Belarus, the Russian Federation and Ukraine[34].

Several studies have been conducted in order to find the potential influence of post-Chernobyl radiation on the health of Ukrainians. For example, Chernobyl Childhood Illness Program (CCIP) examined 116 655 adolescents for thyroid gland disorders and found an increased prevalence of thyroid tumors, including thyroid cancer in this cohort[35], which may result from accumulation of radioactive iodine-131 in the thyroid gland. As regards GI tract diseases, Reshetnikov *et al*[36] reported that the prevalence of IBS in Russian children is as high as 38%, *vs* approximately 10%-15%[37]. Considering the proximity of Chernobyl and the Russian territory, it can be speculated that the post-Chernobyl radiation may be the main trigger. Furthermore, a series of cross-sectional studies on population of Ukrainian children and adolescents with IBS symptoms who live in the area contaminated with radioactive nuclides (60-90 km from Chernobyl) revealed significant abnormalities[38-41]. Namely, the subjects in the study were characterized by a higher level of internal whole body radiation due to ^{137}Cs comparing to control group and exhibited differences in blood parameters, which indicated changes in innate and humoral immune status. In each age group an elevated leukocyte count was detected, with the difference reaching statistical significance in the group of mean age 14. Moreover, a decrease in total T-lymphocyte number, including $CD4^+$ cell population was also observed. The changes were accompanied by an increase in $CD8^+$ level, resulting in significantly lower proportions of $CD4^+$: $CD8^+$ in each group[38]. These observations remain consistent with the theory of low-grade inflammation occurring in some IBS patients[42]. Consequently, Ohman *et al*[43] confirmed the importance of alterations in blood T-cells for generation of symptoms in IBS.

High prevalence of functional GI diseases observed in Ukrainian children and adolescents could also be associated with changes in the non-specific immune system response, resulting from a significantly lower level of neutrophils, but higher level of $CD16^+$ cells in peripheral blood compared with controls. Moreover, a decrease in phagocytic activity and phagocytic index were observed[39]. Altogether, these changes could trigger disturbances in the interaction between host and intestinal microorganisms, resulting in the overgrowth of the latter and/or inflammation, leading to IBS[44]. This hypothesis is supported by the results of a meta-analysis demonstrating the coexistence of intestinal inflammation and IBS in 39% of 1703 studied patients[45]. The impact of the radiation on the development of inflammation-based functional GI diseases in the Ukrainian children can be more complex, since they also present elevated plasma levels of proinflammatory cytokines, such as IL-4 and interferon γ (IFN-γ)[41], what signifies the involvement of several molecular pathways.

The humoral component of the immune response seemed to be altered in the studied population, since the $CD22^+$ B lymphocyte level in peripheral blood was generally increased regardless of the group. In addition, the analysis of the serum immunoglobulin status revealed an increase in IgA, IgG and IgM levels, although the statistical significance was only reached for IgM[40]. Interestingly, several similarities were found between the Ukrainian cohort and a large group of IBS patients from another study[46]. The humoral status of the latter group seemed to be activated, with B lymphocyte and plasma cell density increased in intestinal mucosa compared with that in healthy controls. Moreover, the number of mucosal IgG^+ cells and the luminal concentration of IgG were also higher. Additionally, the density of IgG^+ cells in jejunal mucosa was positively correlated with the number of bowel movements per day and stool form. Whether the activation of humoral immunity is the principal factor in pathogenesis of IBS, remains to be elucidated.

STRESS POLLUTION

The association between stress and physical disorders was observed as early as in the 12th century by Maimonides, a medieval philosopher. He described emotional upset to be an important factor in asthma[47]. Today, we recognize a wide range of diseases with etiology linking mental and somatic disturbances and we call them "psychosomatic disorders". The definition proposed by the World Health Organization states that the psychosomatic disorders are caused by events

in the external environment which evoke responsive brain processes that activate neuro-endocrine systems and thereby induce changes in the functional state of "target" organs and motor systems. The events may play a dominant or only an additional role in the etiology of diseases, along with other factors, such as genetic and nutritional[48]. Such "external" events are often associated with stress and stressful stimuli, which - if in a high number - weaken the organism instead of making it prepared for a challenging situation. Here we propose that exposure to harmful stress can be compared to conventional pollution with regard to its negative impact on human health. Consequently, term "stress pollution" can be used.

Stress is an important contributor to anxiety disorders and thus their prevalence is currently high. For example, a report published in 2001 showed that 5.5% of Australians met the criteria for any anxiety disorder in the past 1 mo and 9.5% in the past 12 mo[49]. A survey conducted between 2001 and 2003 on 9282 Americans demonstrated the lifetime prevalence of anxiety disorders to be as high as 30%[50]. Analysis of 87 studies executed in different countries allows to estimate the current global prevalence of anxiety disorders at about 7.3%[51].

It has been hypothesized that IBS possesses psychosomatic basis and its association with anxiety disorders was therefore investigated. In a group of 94516 participants, in which IBS prevalence rate was 9.7% significantly more anxiety disorders were noted in IBS patients than in individuals free of any functional somatic syndromes[52]. Since median age of onset of anxiety disorders in Americans is 11 years[50], stress during childhood must be one of the most important underlying factors for IBS.

Emotional stress has been proven to induce IBS symptoms also in the adulthood. Lee *et al*[53] studied a group of 23698 subjects (mean age = 48 years) who underwent upper and lower endoscopy; Rome Ⅲ criteria were used to diagnose IBS and The Brief Encounter Psychosical Instrument-Korean version (BEPSI-K) measured severity of stress in patients. More than 26% of participants fulfilled criteria for IBS and the disorder was more common amongst subjects with high stress score. Of note, stress was identified as an independent risk factor for IBS and the disease incidence rate increased along with the stress level. This association could be explained by the analysis of brain-gut interactions elicited by stress. The main culprit, corticotropin-releasing hormone (CRH) secreted by hypothalamus during stressful events can increase intestinal permeability leading to IBS development[54].

The disturbance of the circadian rhythm is another stressor which can determine IBS; furthermore, as such it can be regarded as occupational hazard. Professions particularly exposed to this type of stressor are those with shift work, for example the nurses. Nojkov *et al*[55] studied the prevalence of IBS in nurses based on their working hours. By comparing groups with day, night and rotating shifts it was found that the prevalence in the last group is significantly higher than that in the first one. The association was still significant after adjustment for sleep quality. Since circadian rhythm has been suggested to influence colonic motility in healthy subjects[56], the disruption of the process could have led to IBS development in the studied groups. The association seems to be supported by a clinical trial, which used melatonin, a hormone regulating circadian clock, as a potential new therapeutic in IBS management. Treatment with melatonin improved abdominal pain and distension compared with the placebo-treated group. Noteworthy, the observed effect was not due to the effect of melatonin on sleep patterns, meaning that the improvement in IBS symptoms could have resulted exclusively from the changes in circadian clock[57].

Stress could also influence the development and progression of IBS in an indirect manner. Namely, chronic psychological stress enhances vulnerability to some chemical exposures and in consequence increases the odds for some diseases to develop. The phenomenon has been extensively reviewed by Cooney[58], thus we limit our discussion to the link with IBS. A study investigating the influence of the exposure of rats to urban particles on visceral nociception has revealed an increased vulnerability to abdominal pain[9]. Another study revealed that the exposure to concentrated ambient particles (CAP), which represent modified urban air pollutants, had a more deleterious impact on the respiratory system in stressed rats comparing with non-stressed animals[59]. This means that not only air pollution is able to affect digestive system, but also the grade of its impact would depend on mental status of the animal. In line, epidemiological data collected by Kaplan *et al*[9] have shown that the number of admissions to emergency departments in Edmonton (Canada) due to non-specific abdominal pain was the highest on days when the concentration of polluting gases and solid particles in the air was elevated. Consequently, it can be hypothesized that there is interplay between stress and "conventional" pollution as regards development of functional GI diseases.

Finally, stress related to alteration in nutritional pattern should also be considered as a trigger for IBS. One of the most striking pieces of evidence comes from a study on the post war Dutch population. World War Ⅱ in the Netherlands caused famine which persisted six months till the country was liberated. Daily ratios at that time were about 400-800 cal. Klooker *et al*[60] have investigated the prevalence of IBS in Dutch cohort exposed at the age of 0.5 to 1,5 years to the wartime condition described above and compared it with the prevalence estimated for population conceived after the war had ended; the result was 11% *vs* 8.5% in the post-war generation. It is not clear what caused IBS more prevalent in the patients exposed; however, animal models point at undernutrition as a probable

candidate. In rats subjected to postnatal protein restriction, the growth and mucosal enzyme activity of the GI tract were impaired, what subsequently could have determined functional abnormalities[61].

CONCLUSION

To summarize, the evidence of the involvement of environmental pollution in the development and progression of IBS is very scarce. However, although the role of environmental pollution has not been fully elucidated, available data suggests that it is one of the key factors in IBS pathophysiology. Future research is thus warranted to provide reliable overview of this subject.

UEG scenarios and implication for digestive and liver disease

In October 2014, United European Gastroenterology published a document on Healthcare in Europe: Scenarios and Implication for digestive and liver disease. The main purpose of this release was to draw public attention to digestive diseases, which have become a heavy burden for primary care. Also, the aim of the publication was to raise awareness of current medical school students, who will soon come across this problem in their practice.

Three scenarios were proposed in the publication, namely Ice Age, Golden Age and Silicon Age; each draws a different condition, in which basic and clinical gastroenterology will be in 2040. Here our purpose is to comment on the relation between environmental pollution and functional GI diseases, including IBS according to each scenario.

Ice age

This scenario assumes nature devastation. Economic crisis will lead to negligence in care for environment, which means a higher risk for environmental catastrophes, such as microbiological pollution in Walkerton. In the Ice Age era a significant increase in IBS incidence may be expected. Moreover, unprocessed, healthy and non-polluted alimentation is out of range for middle class, and a widespread antibiotic resistance expands post-infectious IBS toll. An increasing economic gap creates an opportunity for the growth of private healthcare: only the richest receive accurate prevention and only in this group IBS is adequately diagnosed and treated. The underprivileged majority uses public healthcare. Finally, ubiquitous crisis leads to stress pollution, which increases IBS prevalence.

Golden age

In this idealistic scenario, better treatment is provided through better understanding of diseases. The gap between developing and developed countries still exists, but it is diminishing. Environmental protection is thriving, what provides healthy, high-quality and non-polluted food affordable for the majority of human population. Moreover, Europe-wide antibiotic resistance surveillance programs help avoid post-infectious IBS and reduce symptoms from the very beginning, in this way improving general quality of life. Additionally, local food consumption is promoted and chemical and pesticide use is regulated; severity of IBS symptoms is thus lowered. Level-handed politics minimizes everyday-life stress and thus stress-pollution, which also underlie IBS.

Silicon age

It is the most promising scenario. Better tools and procedures ensure better IBS diagnosis and in consequence more IBS cases; however, alleviation of symptoms is more plausible as well, owing to new generation of medications. Finally, more sophisticated technology helps avoid antimicrobial resistance. In this scheme, developing countries overhaul developed countries because of numerous e-medicine solutions. Noteworthy, in this scenario an increase in atomic power usage results in a greater risk of catastrophe; consequently, radioactive caesium (137Cs) leakage, which is a pollutant contributing to IBS development, is likely. On the other hand, automation and overall progress in genetic engineering allow less frequent use of pesticides, what lowers soil pollution with chemicals.

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TOPIC HIGHLIGHT

2015 Advances in irritable bowel syndrome

Food, fibre, bile acids and the pelvic floor: An integrated low risk low cost approach to managing irritable bowel syndrome

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Abstract

Patients presenting with abdominal pain and diarrhea are often labelled as suffering from irritable bowel syndrome, and medications may be used often without success. Advances in the understanding of the causes of the symptoms (including pelvic floor weakness and incontinence, bile salt malabsorption and food intolerance) mean that effective, safe and well tolerated treatments are now available.

Key words: Bile acids; Pelvic floor; Food intolerance; Irritable bowel syndrome; Diarrhoea

Core tip: Decreasing the dietary intake of poorly absorbed carbohydrates and/or using bile acid binders can greatly decrease symptoms of diarrhoea. Pelvic floor weakness with urgency and incontinence may masquerade as diarrhoea and can be managed with soluble fibre supplements and bile acid binders in many cases.

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INTRODUCTION

Patients with functional gastrointestinal disorders dominate the waiting rooms of general practitioners and gastroenterologists alike, and the financial burden of looking after them is considerable[1,2]. When faced with a condition that is of high prevalence, appreciable morbidity but without associated mortality,

low cost well-tolerated treatment options are sorely required. Evolving pharmacotherapies, whilst promising come at significant financial cost[1]. Precise history taking to define dietary indiscretions and adjust intake, particularly of poorly absorbed carbohydrates (FODMAPS) has gained increasing acceptance and is now validated by randomised controlled studies[2]. Some cases of diarrhoea labelled as irritable bowel syndrome with predominant diarrhoea (IBS-D) may, on further questioning represent evolving urgency and incontinence in the context of pelvic floor dysfunction[3,4]. Simple measures again including dietary modification, fibre supplementation and also instructions about toilet habits are effective treatments for many, and supported by clinical studies[5,6]. Finally, for the patient with refractory diarrhoea, manipulation of the bile acid pool with the empirical use of sequestrants such as cholestyramine may be useful, although more studies are needed[7]. Unifying these three broad subject areas (namely the role of dietary intake, the pelvic floor and bile acids in functional symptoms) is a growing awareness of their profound impact on gastrointestinal physiology, the lack of available or reliable investigations, but the simplicity, low cost and low risk of empirical treatment. This opinion-based review considers the rationale and evidence behind these management strategies and presents a pragmatic and cost effective approach to treatment.

FOOD INTOLERANCE AND FUNCTIONAL GI SYMPTOMS

Patients with IBS frequently attribute certain foods as a precipitant to their symptoms. Several recent, high quality reviews have comprehensively outlined this area. Notably, some of the commonest implicated foods are coffee and hot spices (which remain relatively unstudied) but also peas and cabbage (which would be encompassed by the acronym FODMAP). An awareness of these trigger foods obviously opens the door to simple avoidance, a process that obviously requires the patient to accept treatment as opposed to investigation and cure as the goal.

The Low FODMAP approach to the treatment of gastrointestinal symptoms is now well studied and the efficacy is established by a number of studies, albeit usually involving small numbers of patients but notably including randomised and placebo controlled methodologies. The underlying rationale to this approach is that poorly absorbed carbohydrates may exert an osmotic effect in the small bowel (leading to water retention and diarrhoea) and may be fermented in the colon (leading to distension and a feeling of bloating). This is supported by a study of patients with intestinal stoma, where the diet decreased stomal output, and by a separate MRI study of patients with intact gastrointestinal tracts demonstrating both increased small intestinal water content and colonic distension[8,9]. Interestingly, improvements in global satisfaction with gastrointestinal functioning, including constipation as well as diarrhoea are reported by patients, and this strategy has been studied in all subtypes of irritable bowel syndrome[2]. Theoretically however, the removal of osmotically active molecules should worsen constipation.

Investigation of carbohydrate absorption or "malabsorption" utilizing hydrogen breath test (HBT) is proposed to assist in the appropriate selection of individuals likely to respond to dietary restriction of these substrates. However, healthy individuals vary markedly in their ability to absorb carbohydrates such as fructose (commonly tested with HBT), and the reliability of the HBT (including test - retest data) has been brought into question[10,11]. The results of HBT have never been used in a research setting to ascertain a response to dietary modification, with the major studies empirically reducing FODMAP intake. Thus HBT's cannot be recommended as part of the management of patients modifying their FODMAP intake.

Resources are readily and affordably available to help patients manage their IBS symptoms *via* the low FODMAP approach. Applications for smart phones and tablets, websites and cookbooks enable many to self-administer the diet. It is recommended however that a supervised process of graded reintroduction occur to minimise the stringency of the modification, given the evolving evidence that intestinal microbiota are altered, and the potential that products of colonic fermentation (such as short chain fatty acids *e.g.,* butyrate) that would otherwise be produced in a routine diet may be reduced and are physiologically important (this is yet to be demonstrated)[12].

Dietary protein and dietary fat intolerance occur but are less well understood and interventions remain ineffective or unstudied. The protein receiving greatest attention from scientists, patients and the popular press is gluten. The phenomenon of gluten intolerance is controversial and conflicting research abounds[13,14]. Outside of patients with established coeliac disease, many with normal coeliac serology and normal duodenal biopsy following gluten loading (the gold standard) avidly ascribe symptoms to the ingestion of gluten, and attest to improvements on a gluten free diet. It is possible that carbohydrate components of the wheat (fructans) that are poorly absorbed and thus considered FODMAPS are responsible for the symptoms[13]. Alternatively, additives in bread and baking techniques may be the cause of this modern epidemic[15]. Many clinicians speak of patients that can tolerate bread in France or Italy, only to experience symptoms on returning home, a fact that may be secondary to an increased utilisation of fast - rise bread making techniques in countries such as the United Kingdom.

It is likely that dietary fat also is responsible for

Table 1 Types of bile acid malabsorption

Type 1	Examples
	Terminal ileitis (*e.g.*, Crohn's disease)
	Following resection of terminal ileum
Type 2	No definable underlying abnormality
	(this would apply to idiopathic chronic diarrhoea with a response to bile acid sequestrants and/or abnormal SeHCAT)
Type 3	Post cholecystectomy
	Post vagotomy
	Coeliac disease

symptoms in patients with IBS, and that modification may improve symptom control, however this remains unstudied in the context of practical clinical dietary studies. An interventional laboratory based study demonstrated increased rectal sensitivity to balloon inflation induced by duodenal lipid infusion, which provides a compelling argument that lipids are important in IBS, given that sensitivity to rectal balloon distension has been proposed as a surrogate marker for the visceral hypersensitivity that underpins the pathophysiology of IBS[16]. Pancreatic insufficiency and a positive response to pancreatic enzyme replacement has been described in patients with IBS-D, although the evidence for this approach is currently scant[17]. If further research is supportive, then the use of pancreatic enzymes, along with the other measures proposed herewith could in addition offer a low cost, readily available treatment option.

Bile acids and diarrhoea

Clinicians first learnt that bile salts caused diarrhoea by observing patients with Crohn's disease that had undergone ileal resection. Pioneering work by Hoffman *et al*[18], demonstrated increased colonic bile acid exposure, increased stool weight and water content that was reversible when cholestyramine was administered. Similarly diarrhoea induced by cholecystectomy may respond to cholestyramine[19]. In routine clinical practise, we manage many patients that have urgency, abdominal pain, diarrhoea and even occasional incontinence years after cholecystectomy that has passed unrecognised by other practitioners. Typically these patients respond to cholestyramine. In recent years, the proposition that anatomically normal individuals may have measurable abnormalities in bile salt recirculation has gained acceptance[20]. The proposed subtypes of bile acid malabsorption (BAM) are presented in Table 1.

BAM may be a more appropriate diagnosis in at least 25% of patients with IBS-D, and treatment with a bile acid binder may improve the symptoms of many patients with unexplained diarrhoea with (or perhaps more controversially) without BAM demonstrated by selenium homocholic acid taurine (SeHCAT)[21]. In the future, the use of BAS may not be limited simply to treating diarrhoea, and have been trialled for patients with incontinence, anorectal pain post haemorrhoidectomy and for gastritis post cholecystectomy[22-24].

Bile salts are excreted from the liver and are involved in the solubilisation and lipolysis of ingested lipids, thus facilitating absorption in the small intestine[25]. The conjugation within the liver of the bile acids to glycine and choline to produce chenodeoxycholic acid and cholic acid allows them to remain in an ionised form that resists passive absorption. Rather, 95% of excreted bile acids are absorbed *via* the apical Na^+ dependent transporter in the ileum. The process whereby bile acids are produced in the liver, stored in the gallbladder, released into the duodenum and absorbed in the terminal ileum is termed the enterohepatic circulation of bile acids[26] (Figure 1).

The regulation of bile acid production and recirculation involves a negative feedback loop where the receptor farnesoid X (FXR) in the ileum and liver senses the recirculated bile and, *via* secondary mechanisms involving gene transcription and production of the inhibitory fibroblast growth factor-19 (FGF-19), leads to decreased bile acid synthesis from cholesterol (a more detailed discussion can be found elsewhere as listed)[20].

The delivery of excess amounts of the bile acids chenodeoxycholic acid and deoxycholic acids to the colon results in excess salt and water excretion, colonic contractions and thus potentially diarrhoea whilst a deficiency may have the opposite effect and cause constipation[27]. These observations arguably should place interventions related to bile acid delivery to the colon at the forefront of considerations when treating these symptoms (see below).

The suggestion that many patients with IBS-D have BAM means that a large number of current patients have an undiagnosed, undefined and untreated entity. The alternative view is that modulation of bile acid recirculation with bile acids sequestrants will alter intestinal transit in most patients, with the results of investigations to delineate physiological variation instead arbitrary, untested and not useful. From a theoretical standpoint, excess conjugated bile acid delivery to the colon could be secondary to: (1) Excessive bile salt production; (2) Inefficient bile salt resorption (due to abnormalities of active transport mechanisms in the ileum or rapid transit precluding adequate absorption); (3) Excessive colonic salt and water production, or colonic motility in contact with a "normal" amount of bile salts; and (4) Abnormal bile salts.

The preferred explanation for bile salt diarrhoea, is in fact, excessive production of bile salts due to a failure of the negative feedback loop, as a consequence of inadequate FGF-19 production[28]. An enlarged bile acid pool thus causes diarrhoea, and supposedly would cause an abnormal SeHCAT test[7,29]. Expansion of the bile acid pool in those with clinical BAM has been previously demonstrated. Conflicting

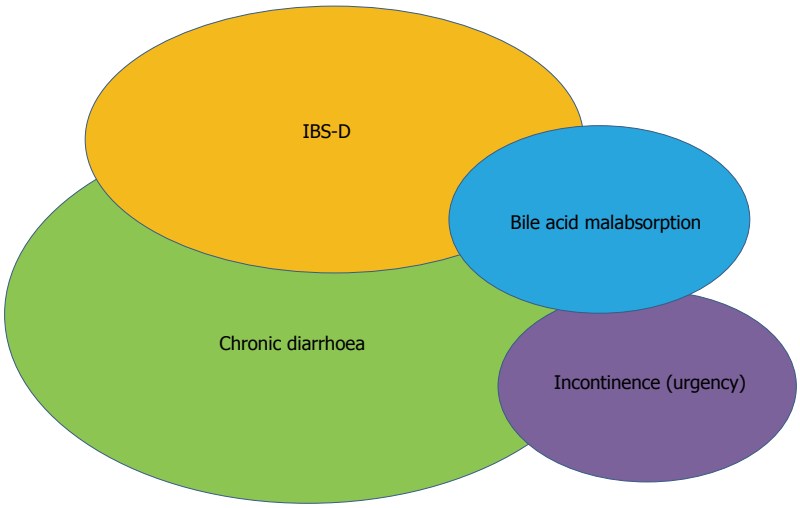

Figure 1 Overlapping entities presenting as loose motions and abdominal discomfort. Note that patients with incontinence and urgency often report abdominal pain, as do those with loose motions and a positive SeHCAT. IBS-D: Irritable bowel syndrome-diarrhea.

data emerge when attempting to correlate SeHCAT values and FGF-19, with a recent study failing to demonstrate a difference between healthy controls and those with IBS-D[28]. Earlier research however has linked low FGF-19, elevated plasma 7 alpha-hydroxyl-4-cholesten-3-one (C-4 - a surrogate marker of the hepatic biosynthesis of bile) and BAM[25].

Inefficient bile acid absorption is thought to be rare, with abnormalities of genes coding the ileal apical bile acid transporter thought to be uncommon, phenotypically rare and limited to well defined familial cases[30]. Rapid small intestinal transit may explain BAM, although this theory is only weakly supported by evidence and the high efficiency of the apical BA would make this hypothesis less likely[31]. The notion that the SeHCAT may instead reflect alterations in small intestinal transit is also disputed with contradictory evidence[32].

The response of the animal colon and importantly human colon to bile acids has only been studied in several small experiments, and further more definitive enquiry seems technically difficult and unlikely to occur. However it seems plausible that significant differences between individuals when exposed to the same concentration of bile salts could occur. Variations in the constituents of bile salts have not been studied in this context.

INVESTIGATION

Selenium homocholic-acid taurine (SeHCAT) is a radiopharmaceutical that is licensed for the investigation of BAM, and demonstrates behaviour identical to endogenous bile acids once being absorbed in the ileum after oral ingestion[7]. The severity of the BAM (or loss) is defined when measured by a gamma camera at 7 d and is defined by the percentage remaining *in-situ* (cut off commonly < 5% defining severe malabsorption, and 15% mild). SeHCAT is the only widely available measure of enterohepatic bile salt recirculation, and as such is used as to define the entity of BAM. There is no comparable test commonly available, and no gold standard definition of BAM. The flaw is this approach is that normal values have never been clearly established, that the reliability of the test (including test-retest characteristics) remains unknown, and that the definition of an abnormal result varies. A health technology review commissioned by the National Health Service (NHS) in 2013 concluded that before SeHCAT can be recommended as a reliable and cost effective measure, studies that include treatment of all patients regardless of SeHCAT result are needed[33]. The majority of those performed to date have simply compared patients with variable levels of BAM, with retention of selenium isotopes of < 5%, < 10% or < 15 % respectively most often reported. In fact in a recent systematic review of the area, the efficacy of patients treated with cholestyramine did not differ between those with various levels of BAM defined by SeHCAT[21].

Several alternative tests are available that hold future promise in defining BAM, but require further validation. Serum C-4 is a surrogate measure of bile acid production, whilst FGF-19 is produced by enterocytes and hepatocytes and is integral to the negative feedback loop regulating bile acid production[34]. Studies have variably demonstrated a correlation between these seromarkers and the SeHCAT, and clinically with symptoms of BAM[20]. Measurement of stool bile acids *via* spectroscopic techniques is feasible but costly.

TREATMENT

In countries such as the United States and in Australasia, SeHCAT is either not available or available in a limited capacity. This means that empirical treatment with bile acid binders such as cholestyramine

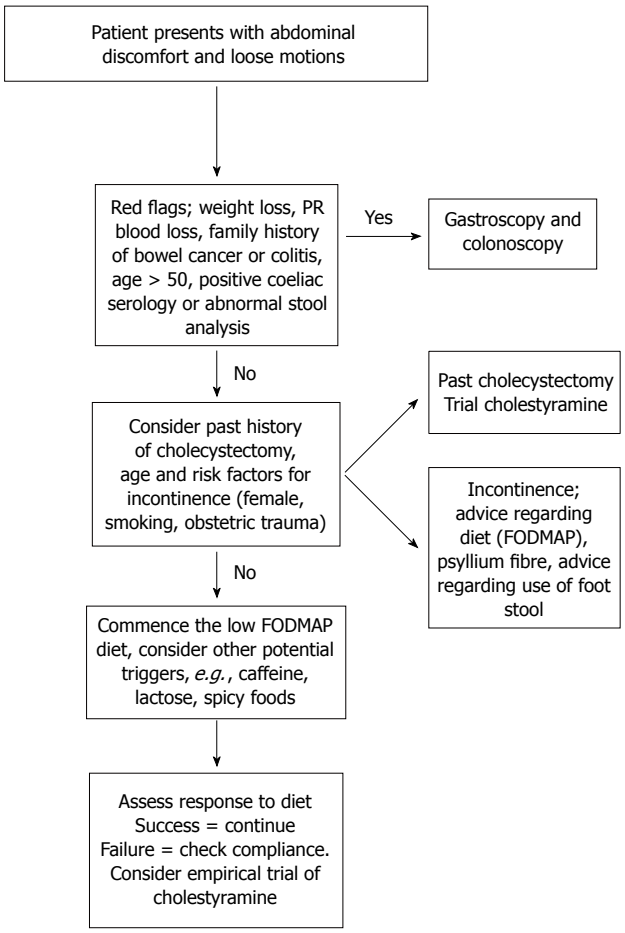

Figure 2 Proposed management algorithm for patients with loose motions ± abdominal pain.

is the only rational and available approach, whereby a response to treatment defines the entity. There is much to commend this approach given the current concerns with the validity of measures for BAM, although in the context of an aim to improve, tailor and streamline the treatment of patients with functional GI conditions this is questionable.

There is little doubt in our minds, based on the available evidence and our own clinical experience, the bile acid sequestrants have a potent effect on gastrointestinal physiology and thus ameliorate symptoms of diarrhoea in many patients. Some degree of caution should be exercised before widespread empirical therapy can be proposed first line in those with diarrhoea however. We thus propose this treatment in those who have not responded to dietary therapy (Figure 2). The ability of cholestyramine to ameliorate symptoms of IBS-D in a patient with a normal SeHCAT is unknown, and even when there is a clearly defined abnormality on SeHCAT, open studies (as opposed to placebo controlled) only have been undertaken. Cholestyramine is not always well tolerated, with 20%-30% of patients ceasing this medication as a result, describing unpleasant taste, constipation (ironically) or abdominal discomfort[21]. Newly developed medications such as colesevelam, and a colonic release colestyramine may improve palatability and limit side effects, however limited availability and high cost limit these options currently[35]. Finally, malabsorption of fat soluble vitamins has been described, suggesting that monitoring with blood tests (*e.g.,* INR and vitamin D) and supplementation where necessary (*e.g.,* multivitamins) is needed[36,37].

Defecatory disorders

Disorders of defecation are a common cause of gastrointestinal symptoms[4]. Awareness of and training in these issues is arguably suboptimal within the field of gastroenterology[38]. Several recent comprehensive position papers and reviews have emphasised the importance of considering these conditions early in the management of patients with altered bowel habit[39,40].

Incontinence and urgency

Faecal incontinence, defined as the unintentional passage of faeces or flatus encompasses a range of severities, and is a common and socially debilitating problem, affecting 5%-15% of adults, with some but not all studies demonstrating a female gender predominance[41]. Other risk factors for faecal incontinence include cigarette smoking, multiparity, advancing age and cholecystectomy. Demonstrable deficits in anal sphincter integrity (*e.g.,* at endoanal ultrasound) are particularly prevalent in multiparous women, sphincter atrophy common in smokers and pudental nerve injury is a factor in some cases[6].

A full, detailed knowledge of the investigations available to diagnose FI is not practically required for most gastroenterologists (and indeed is the domain of colorectal surgeons in many regions of the world). An awareness of the spectrum of the problem (that may be described by patients as "diarrhoea" until questioned further) and the good response to conservative treatments in 25%-50% is however vitally important[41]. A range of incontinence scoring systems have been proposed, and it is notable that included in some definitions is an inability to defer defecation for 15 or more minutes, (hence urgency is included) and the use of anti-diarrhoeals[42]. In clinical practise we are referred many patients who are mildly incontinent but have had the diagnosis missed or labelled as diarrhoea. Urgency and lower abdominal discomfort is often a feature. Obviously, exclusion of other conditions or precipitants (that may affect particularly women in their 5th and 6th decade- the age when incontinence has a peak onset in adults) including microscopic colitis and BAM type 3 is also essential.

Conservative therapies including dietary advice (eliminating the ingestion of poorly absorbed carbohydrates), use of bulk forming laxatives (in particular psyllium), instruction concerning the most efficient

posture to defecate (with the feet elevated at least 10 cm from the floor to open up the anorectal angle) and possibly pharmacotherapy (loperamide is most frequent, but interestingly cholestyramine has been trialled and found to be effective in an open labelled study, as has clonidine) have in combination demonstrated efficacy[24,43]. Failure of these simple measures mandates referral for physiological and anatomical investigations including anorectal manometry and endoanal ultrasound.

CONCLUSION

The symptoms of abdominal pain and diarrhoea are common causes of morbidity. Simple low risk high efficacy treatments are available. Dietary modification of poorly absorbed carbohydrates is a strategy now supported by randomised controlled trial evidence. Incontinence with pelvic floor weakness (often misdiagnosed as diarrhoea) is effectively managed conservatively in many. Evolving evidence suggests widespread use of empirical bile acid sequestrants may be appropriate in unexplained diarrhoea unresponsive to dietary modification.

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TOPIC HIGHLIGHT

2015 Advances in Pancreatic cancer

Molecular detection of pancreatic neoplasia: Current status and future promise

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Abstract

Pancreatic cancer is usually diagnosed at an advanced stage and curative resection is feasible in only a small minority of patients at the time of diagnosis. Diagnosis at an early stage is unequivocally associated with better long-term survival. Several candidate molecular markers for early detection are currently under investigation in different phases of discovery and validation. Recent advances in the technology for whole genome, methylome, ribonucleome, and proteome interrogation has enabled rapid advancements in the field of biomarker discovery. In this review we discuss the current status of molecular markers for detection of pancreatic cancer in blood, pancreatic cyst fluid, pancreatic juice and stool and briefly highlight some promising preliminary results of new approaches that have the potential of advancing this field in the near future.

Key words: Early detection of cancer; Sensitivity and specificity; Pancreatic cancer; Pancreatic juice; Stool; Pancreatic cyst fluid; Biomarkers; Methylation

Core tip: Pancreatic cancer is a leading cause of cancer mortality worldwide. Early detection at a resectable stage is associated with the best long-term prognosis. There are ongoing efforts globally to discover, validate and optimize molecular markers for early diagnosis. The challenge is to develop highly sensitive markers not only for earliest stage cancer but also to accurately detect premalignant lesions with high grade dysplasia that would maximally benefit from resection. In this review, we summarize some of the most promising biomarkers for molecular detection of pancreatic cancer and discuss evolving molecular approaches.

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INTRODUCTION

In 2015, an estimated 48960 people will be diagnosed with pancreas cancer (PC) in the United States[1]. Based on current SEER (Surveillance, Epidemiology, and End Results Program) statistics factsheets published by the National Cancer Institute (NCI), only a small minority of them (7.2%) will survive for 5 years or more[1]. In 2012, 337872 new cases of PC were reported worldwide along with 330372 PC-related deaths[2]. PC is currently the seventh leading cause of cancer mortality worldwide and is projected to become the second leading cause of cancer mortality in the United States by 2030[3,4].

Over the past two decades the 5-year survival rate of PC has improved from 3.6% to 7.2%[1]. This has been attributed to improvements in peri-operative care and decreased surgical mortality[2,5]. However, definitive surgical intervention is feasible in only a small minority of patients diagnosed with PC. The disease is localized to the pancreas in less than 10% cases at the time of initial diagnosis and patients with localized disease have a significantly better outcome with 5-year survival rate of 27%[1]. In instances of incidentally discovered asymptomatic stage I disease, 5-year survival may exceed 70%[6]. It is obvious that diagnosing PC in the early stages will significantly improve survival.

There is an urgent need to develop early detection methods to improve these outcomes. New molecular approaches offer the promise of accuracy, ready distribution, and affordability that will be needed to deliver practical screening tools. For a diagnostic biomarker to be clinically useful in early detection of PC, the most important characteristics are high sensitivity, high specificity, and ability to discriminate low-grade dysplasia from high-grade dysplasia and early cancer. Such performance features would help select patients for early endoscopic or surgical intervention with curative intent. Highly sensitive markers that lack specificity can result in a large number of false positive tests and lead to unnecessary and expensive tests and procedures.

BIOLOGICAL CONSIDERATIONS

Contrary to common belief that pancreatic ductal adenocarcinoma (PDAC) is a rapidly-growing malignancy, its progression through the stages of precancer to metastatic disease may take an average of two decades[7]. This is a critically important observation suggesting that there may be an ample period during which to screen the key target lesions-earliest stage cancer and those precancers at greatest risk of progression[8].

Although the earliest precancer lesions in PDAC, also known as pancreatic intra-epithelial neoplasia (PanIN), are well-defined from a histologic standpoint, currently available imaging techniques and biomarkers lack sensitivity for their detection. The cystic premalignant lesions which include intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MCNs) are more readily identifiable by imaging. However, as the large majority of these cystic lesions do not progress to cancer, their incidental detection often results in a management dilemma. Current risk prediction models are imperfect and the proportion of pancreatic resections for non-neoplastic lesions appears to be much higher than the proportion of "missed" cancer[9].

Thus, the natural history of PDAC appears to present a window of opportunity for detection of early stage and potentially-curable neoplasms. The challenge is to develop highly sensitive molecular markers not only for earliest stage PDAC but also with the ability to accurately detect pancreatic premalignant lesions with high grade dysplasia that would maximally benefit from surgical resection.

ADVANCES IN TECHNOLOGY

The past decade has witnessed explosive progress in whole genome, methylome, ribonucleome, and proteome interrogation that has accelerated the identification of candidate biomarkers for early detection of PDAC. While candidate markers generated from these discovery engines have potential to yield discriminant tests when applied to distant media, clinical validation and test development are largely at early stages. The use of a variety of assay platforms has been explored on diverse media such as plasma, serum, cyst fluid, pancreatic juice and stool. Molecular markers have been evaluated to detect both cancer and premalignant pancreatic lesions, assess prognosis, and predict tumor response to specific pharmacotherapy. In the future, such molecular tests may be applied to guide individualized cancer therapy.

Over the past decade, several molecular tests have been studied in pancreatic neoplasms (Table 1). In this review, we describe the molecular markers that are currently in the pipeline and also discuss evolving molecular approaches which may potentially alter the diagnostic paradigm for pancreatic cancer in the future. The focus is on PDAC and cystic neoplasms of the exocrine pancreas since these comprise the majority of malignant pancreatic tumors.

Blood testing

Conventional approach: Conventional biomarkers for the detection of PDAC have been largely protein-based and have several limitations. Carbohydrate antigen 19-9 (CA 19-9) is the most widely studied conventional biomarker for PDAC. Importantly, levels are often normal in early disease and falsely elevated in patients with various conditions, such as biliary obstruction[10]. Furthermore, it is well known that up

Table 1 Biomarkers for molecular detection of pancreatic cancer

Bio specimen	Type of biomarker	Examples of molecular markers
Blood	Conventional protein markers	Carbohydrate antigen 19-9 (CA 19-9)
		Carcinoembryonic antigen (CEA)
	Novel proteins	Intercellular adhesion molecule-1 (ICAM-1)
		Osteoprotegerin (OPG)
		Macrophage inhibitory cytokine-1 (MIC-1)
		Tissue inhibitor of metalloproteinases-1 (TIMP-1)
		S100 calcium-binding protein P (S100P)
	Mutated genes	*KRAS, TP53, SMAD4, CDKN2A, KDM6A, PREX2*
	Aberrantly methylated genes	*p16, ppENK, cyclin D2, SPARC/osteonectin SOCS-1, TSLC1*
	Micro-RNAs	miR-1290, miR-145, miR-150, miR-223, miR-636, miR-26b, miR-34a, miR-122, miR-126, miR-145, miR-150, miR-223, miR-505, miR-636, miR-885.5p.
	Circulating tumor cells	(molecular markers not yet interrogated)
Cyst fluid	Mutated genes	KRAS, GNAS
	Aberrantly methylated genes	*BNIP3, PTCHD2, SOX17, NXPH1 and EBF3*
	Micro-RNAs	miR-138, miR-195, miR-204, miR-216a, miR-217, miR-218, miR-802, miR-155, miR-214, miR-26a, miR-30b, miR-31, and miR-125
Tumor tissue	Novel proteins	Gelsolin, Lumican, Galectin-1 and Laminin
Pancreatic juice	Mutated genes	*KRAS, TP53*
	Aberrantly methylated genes	*ADCY1, CD1D, BMP3*
Stool	Mutated genes	*KRAS, BMP3*

to 15% of individuals with a high tumor burden have normal or undetectable CA 19-9 levels. About 5% of Caucasians lack the Lewis blood group antigen and have undetectable levels of CA 19-9 since they lack the characteristic fucosylation pattern required for CA 19-9 detection by commercially available assays[10]. The combination of these factors makes it an unreliable screening tool. Its scope in current practice is largely restricted to detection of tumor recurrence after surgical resection[11,12]. Carcinoembryonic antigen (CEA) has also been studied as a diagnostic test for PDAC and found to have poor performance characteristics. In isolation, it has low diagnostic accuracy for aggregate stages of PDAC with a sensitivity of 54% and specificity of 79%; these respective metrics change to 86% and 72%, when CEA is used in combination with CA 19-9[13,14].

Novel proteins: A variety of discovery approaches have been pursued to profile the pancreatic cancer proteome and identify biomarkers for diagnosis. Several sample types, including whole tumor tissue, isolated neoplastic cells and isolated tumor stromal cells have been used in these discovery and validation efforts[15]. In addition to tissue profiling, the proteome has also been studied in pancreatic juice and serum using liquid chromatography tandem mass spectrometry as well as high-throughput immunologic proteomic strategies[16]. In the past, antibodies to osteoprotegerin, intercellular adhesion molecule-1 (ICAM-1) and tissue inhibitor of metalloproteinases-1 (TIMP-1) have been proposed as potential diagnostic biomarkers for PDAC. In a case-control study of 333 patients with PDAC, a combination of CA 19-9, ICAM-1 and OPG antibodies was found to have a sensitivity and specificity of 88% and 90%, respectively, for distinguishing PDAC from healthy controls[17]. However, a more recent case-control study evaluating ICAM-1 and TIMP-1 levels argued against their utility in the early diagnosis of PDAC. In this study investigators tested the levels of these proteins in pre-diagnosis samples of patients 0-12 mo before a diagnosis of PDAC and a quantitative comparison with non-cancer control samples revealed no significant difference[18]. Interestingly, patients with jaundice secondary to both benign and malignant etiology had elevated circulating levels of these proteins leading the investigators to believe that failure to account for biliary obstruction may have contributed false positive results in prior studies. Other circulating proteins that have been identified as candidate biomarkers include macrophage inhibitory cytokine-1 (MIC-1) and S100 calcium-binding protein P (S100P). Serum MIC-1 may have a higher sensitivity (62.5% *vs* 25.0%) and similar specificity compared to CA19-9 for detection of early stage PDAC[19]. A meta-analysis evaluating the diagnostic performance of S100P reported pooled sensitivity and specificity of 87% and 88%, respectively, for aggregate stages of PDAC with an AUC of 0.93[20].

Further investigation is clearly indicated to corroborate early observations with these novel differentially-expressed candidate biomarkers and to better define their biological significance and clinical utility.

DNA: Mutations - The genomic landscape of PDAC is complex. In recent years, in-depth analysis of the coding region of the genome using high-throughput studies of gene expression has revealed a large number of gene expression abnormalities associated with PDAC. In one of the early studies, Jones *et al*[21] performed SAGE (serial analysis of gene expression) analysis of 24 PDACs with tumor cells passaged *in vitro*

as cell lines or in nude mice as xenografts. This study identified 10-fold overexpression of 541 genes in more than 90% of the tumors when compared to normal pancreatic duct cells[21].

Commonly mutated genes that characterize PDAC include *KRAS*, *TP53*, *SMAD4* and *CDKN2A*. A recently published study describing deep whole-genome sequencing and copy number variation (CNV) analysis of 100 patients with PDAC has reaffirmed that chromosomal rearrangement in these four genes is an important mechanism of DNA damage in pancreatic carcinogenesis[22]. Activating mutations in *KRAS* were identified in nearly all patients in this study and the prevalence of inactivation events for the other genes was 74% for TP53, 35% for CDKN2A and 31% for SMAD4. This study also identified inactivating mutations in two new genes KDM6A and PREX2, occurring in 18% and 10% of patients[22].

The reported mutation rate of KRAS in PDAC ranges from 75%-95%, making it the most commonly mutated gene in PDAC[23,24]. Although KRAS mutation testing appears to be an attractive diagnostic target, commercially available plasma assays lack specificity and are relatively insensitive in the detection of early PDAC[25]. KRAS mutations are known to be present with greater frequency in smokers and in patients with chronic pancreatitis both of which are risk factors for PDAC and further confound the diagnostic utility of this marker[24]. A recent meta-analysis of 8 prospective studies assessing the accuracy of KRAS gene mutation analysis for diagnosing of PDAC reported a pooled sensitivity and specificity of 88.7% and 92% for KRAS mutation analysis combined with cytology of an endoscopic ultrasound (EUS)-guided fine needle aspirate (FNA) compared to 80.6% and 97% for EUS-FNA alone. The authors concluded that there may be a role of using KRAS as a diagnostic marker in cases where the cytology is inconclusive[26]. Recent interest has focused on quantification of KRAS mutants in blood of patients with PDAC as a marker of early diagnosis[27,28]. Kinugasa *et al*[28] recently demonstrated that the ratio of mutant to wild type KRAS could be used as a biomarker in early PDAC.

Although currently available data clearly demonstrate that genetic mutations and alterations in gene expression unequivocally contribute to pancreatic carcinogenesis, none of these genomic markers have demonstrated favorable performance characteristics as a diagnostic biomarker in clinical application. Furthermore, the numerous mutational sites across involved genes render impractical their use as clinical biomarkers with current assay platforms. The evolution of high-speed sequencing platforms could overcome this limitation in the future.

Another challenge of detecting tumor DNA in blood pertains to their very low circulating concentrations at the earliest stages of cancer. Also, unlike RNA and various protein markers, there is typically only one copy number of a given DNA marker per tumor cell. Accordingly, assay platforms with exquisitely high analytical sensitivity are required to render this approach feasible. The availability of digital PCR, next-generation sequencing, and other innovative platforms in recent years may provide the requisite performance to detect very low levels of circulating mutant DNA[29]. These results are preliminary and need to be validated in larger studies.

Methylation - Several studies have focused on the methylation status of PDAC and cystic pancreatic neoplasms. Genes that have been identified to undergo aberrant methylation during pancreatic carcinogenesis include p16, ppENK, cyclin D2, SPARC/osteonectin SOCS-1 and TSLC1[30-35]. Genome-wide study of DNA methylation patterns in 167 resected untreated PDACs demonstrated enrichment of various aberrantly methylated genes, some of which appear to be highly discriminant for PDAC as single markers[36]. Our group has also identified novel methylated DNA marker candidates *via* whole methylome interrogation and has demonstrated the potential utility of methylation markers in site-prediction of gastrointestinal malignancies[37]. The use of these epigenetic alterations as early detection markers for PDAC is encouraging, and rigorous assessment of their application to distant media is now needed.

RNA: Micro-RNAs are non-coding RNAs that regulate posttranscriptional gene expression. The increasingly recognized role of micro-RNAs in oncogenesis and tumor metastasis has been described[38,39]. MicroRNA profiles specific for PDAC have been found in serum, pancreatic tissue, cyst fluid and more recently in whole blood[40-43].

Some of the earlier studies focused on serum and plasma microRNA profiles to distinguish patients with PDAC from healthy volunteers. Li *et al*[40] reported that serum miR-1290 levels appeared to have an excellent discriminatory ability (AUC of 0.96) to distinguish patients with early pancreatic cancer cases from healthy controls. Corroboration and extension of these early observations are needed. Several other investigators proposed the role of other serum microRNAs but most of these early studies lacked independent validation. The Danish BIOPAC (Biomarkers in Patients with Pancreatic Cancer) study analyzed microRNA expression in whole blood in 409 patients with pancreatic cancer. This prospective case-control study identified 2 miRNA panels consisting of sets of 4 (miR-145, miR-150, miR-223, miR-636) and 10 mi-RNAs (miR-26b, miR-34a, miR-122, miR-126*, miR-145, miR-150, miR-223, miR-505, miR-636, miR-885.5p) that distinguished patients with PDAC from healthy controls[41]. The larger panel had a comparatively higher AUC for detection of early pancreatic cancer (AUC of 0.91 *vs* 0.80). Although currently available studies have focused on identifying free miRNA in plasma or serum, there is growing interest in exploring circulating exosomes as a

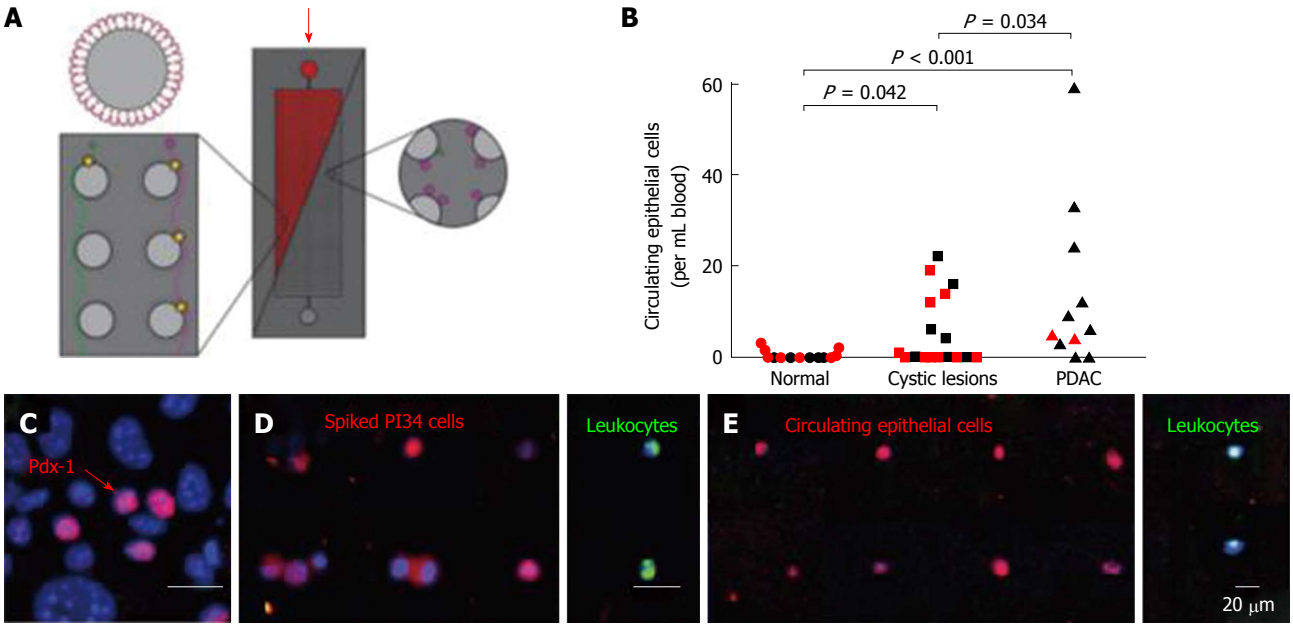

Figure 1 Detection of circulating pancreatic epithelial cells using geometrically enhanced differential immunocapture in cystic and solid pancreatic neoplasms using cellular stains for CD45 (green), Pdx-1 (red), and DNA (DAPI, blue). Scale bar: 20 mm. Reprinted from Rhim *et al*[29].

concentrated and potentially more discriminant source of miRNA in patients with PDAC[44,45].

As is evident from the above data, a large number of mi-RNAs have been implicated as potential biomarkers. The widespread availability of next generation sequencing techniques has facilitated rapid advancement in this field of research. Next steps must involve validation of some of the more promising markers in larger cohorts, especially focusing on discriminating between low-grade and high-grade dysplasia.

Circulating tumor cells: Recent data suggest that hematogenous spread of pancreatic tumor cells may be an early event during pancreatic oncogenesis and circulating tumor cells may be detectable before the primary tumor can be visualized on any imaging test. In mouse models, Rhim and colleagues demonstrated that an epithelial to mesenchymal transition of PDAC tumor cells occurs as an early phenomenon, even before histologic emergence of cancer[46]. Additionally, they used cell lineage labelling techniques in transgenic mice with PDAC for a proof of concept study to show that pancreatic epithelial cells may enter the circulation prior to tumor formation, and followed that by observing the same phenomenon in a subset of patients with cystic pancreatic lesions (Figure 1)[29,46]. Circulating epithelial cells were also detected in 8 of 11 (73%) patients with PDAC, but in 0 of 19 control patients without cysts or cancer. These results are preliminary and need to be validated in larger studies. Other studies have explored the possibility of using circulating tumor cell detection as a marker of predicting prognosis after curative surgical resection of the primary tumor. Based on preliminary data circulating tumor cell testing appears to be more sensitive (75% *vs* 69%) and specific (95% *vs* 81%) than CA 19-9 for diagnosing recurrent disease[47].

Cyst fluid and tissue testing

Advances in endoscopic ultrasound have provided a relatively safe modality for tissue acquisition from malignant and premalignant pancreatic lesions. Several investigators have studied fine-needle aspirate and cyst fluid specimens for biomarker detection.

The multicenter PANDA (Pancreatic Cyst DNA Analysis) study found the combined presence of a KRAS mutation and allelic loss in cyst fluid to have a sensitivity of only 37% at a specificity of 96% for discriminating malignant from benign pancreatic cysts[48]. In another study by Wu *et al*[49], cyst fluid GNAS mutations were detected exclusively in IPMNs and not in any other mucinous, non-mucinous or malignant pancreatic cysts. Hong and associates identified hypermethylation of several genes including BNIP3, PTCHD2, SOX17, NXPH1 and EBF3 in cyst fluid from IPMNs with high-grade dysplasia[50]. In a study comparing the miRNome of low-grade and high-grade pancreatic cystic neoplasms, thirteen miRNAs (miR-138, miR-195, miR-204, miR-216a, miR-217, miR-218, miR-802, miR-155, miR-214, miR-26a, miR-30b, miR-31, and miR-125) were differentially enriched in cyst fluid samples from patients with PDAC[42]. Matthaei and colleagues identified a panel of 9 mi-RNAs in cyst fluid and microdissected formalin-fixed, paraffin-embedded (FFPE) tissue from surgically resected IPMN specimens, which distinguished potentially malignant cysts requiring surgical intervention from benign cysts that could be managed conservatively with a sensitivity of 89%, a specificity

of 100%, and AUC of 1[43]. Such high discrimination in this early report clearly warrants further exploration.

Mass spectrometric analysis of whole tumor tissue has identified several proteins associated with pancreatic oncogenesis. Gelsolin and lumican are two such proteins initially identified in cancer tissue that were found to have 80% sensitivity and 95% specificity as a plasma biomarker in discriminating early stage pancreatic cancer patients and healthy controls[51]. A quantitative proteomic study of PanIN 3 lesions identified more than 200 dysregulated proteins, the majority being cytoskeleton proteins involved in cell motility. Galectin-1 and laminin were overexpressed both in these advanced PanIN lesions and the adjacent pancreatic stroma[52]. Amato *et al*[53] used next-generation sequencing to assess mutations in 48 surgically resected IPMNs and identified GNAS (79%) and KRAS (50%) to be most commonly affected genes. These studies using surgical specimens are susceptible to selection bias and the detection of these genetic mutations is not ideally suited to be applied to the diagnostic algorithm of cysts that do not meet currently established criteria for surgical resection.

Our understanding of the progression model of pancreatic cancer has improved significantly over the past decade. The term pancreatic intraepithelial neoplasia (PanIN) was introduced in 1999 to describe ductal lesions that are precursors of invasive cancer[54]. Although high-grade ductal precursor lesions (PanIN-3) have a greater malignant potential, low-grade PanINs (PanIN-1 and PanIN-2) are more common; especially in older adults and do not always progress to cancer. Thus, biomarkers targeting PanIN-3 would be of greatest clinical utility for early detection. The overexpression of HER-2/neu and point mutations in the K-ras gene appear to be early events that discriminate low-grade ductal premalignant lesions (PanIN-1) from normal ductal epithelial cells[54]. Inactivation of the p16 gene appears to be more common in higher-grade PanINs compared to low-grade PanINs and probably occurs at an intermediate stage of tumorigenesis[54]. Inactivation of p53, DPC4, and BRCA2 are relatively terminal events in this neoplastic progression[54]. Gene expression profiling analyses of cells from normal pancreatic ducts, PanINs and PDAC have revealed more than 1,000 molecules including S100P, MMP7, MUC4, FSCN1, and MUC5AC that are preferentially expressed both in PDAC and precursor lesions[55]. More work is needed both at the discovery level and with subsequent validations to make meaningful progress in identification of useful markers for PanIN-3.

Pancreatic juice testing

Recent studies have focused on analysis of pancreatic juice sampled endoscopically from the duodenum following intravenous administration of secretin to stimulate pancreatic excretion and facilitate juice collection. In one study KRAS mutations were detected in pancreatic juice of 73% of patients with PDAC[56]. However, KRAS mutations were detected in pancreatic juice from 19% of controls and attributed to the presence of small pancreatic intraepithelial neoplasia (PanIN) lesions[56]. Detection of mutant TP53 has been studied in pancreatic juice from patients with both cancer and precancerous lesions in an enriched patient population. Although the sensitivity of this test was only 67.1% in patients with invasive PDAC, TP53 mutations were not identified in any of the 58 controls or in patients with PanIN-1 lesions[57]. In our recent early study using non-optimized techniques on archival pancreatic juice samples, we identified a panel of novel methylated DNA markers and mutant *KRAS* in patients with PDAC. At specificity cutoffs of 90% and 95%: this combined marker panel achieved sensitivities of 88% and 77% for diagnosis of PDAC; *ADCY1*, was the most sensitive single methylation marker. Overall discrimination between PDAC and controls by area under ROC curve was 0.91 for the panel which was significantly higher than by any single marker ($P < 0.05$), except ADCY1. Positivity rates were substantially lower in patients with chronic pancreatitis compared to those with PDAC for all markers ($P < 0.0001$)[58]. With optimization of marker selection, sample processing, and assay techniques, analysis of pancreatic juice has potential to characterize indeterminate pancreatic lesions with high accuracy.

Stool testing

Although considerable attention has been devoted to discovery of biomarkers in pancreatic juice and cyst fluid, the acquisition of these specimens are dependent on invasive procedures and would not be suitable for general screening. In a recent study we explored the possibility of detecting DNA markers in stool as an approach to the early detection of PDAC[59]. Nine target genes were assayed comparing stool samples from patients with PDAC compared to controls with normal colons. BMP3 was the most discriminant methylation marker in stool. At 90% specificity, the combination of methylated BMP3 and mutant KRAS in stool detected 67% of PDACs. AUC for the combination in stool was 0.85, which was better than the AUC for either test in isolation. Further studies are necessary to improve the discriminatory accuracy of stool methylation markers in patients with early PDAC.

FUTURE CHALLENGES AND OPPORTUNITIES

Several additional approaches have recently surfaced which offer creative strategies to early detection of this lethal cancer.

Exosomes are extracellular vesicles containing proteins and nucleic acids surrounded by a lipid-bilayer

wall. Isolation of specific cancer-cell derived exosomes has been studied as a tool for early diagnosis. In a recently published study, circulating cancer-cell derived exosomes enriched in glypican-1 were identified as a marker for diagnosis of PDAC with reported 100% specificity and 100% sensitivity (AUC of 1.0) for detecting PDAC[60].

The pancreas has a major role in regulation of metabolism in healthy individuals. It follows intuitively that metabolomic profiling of individuals affected by PDAC could reveal diagnostic clues. A study using serum assay in a p48-Cre/LSL-KrasG12D mouse model identified a distinct metabolic pattern that distinguished animals with early stage PDAC from wild-type controls with an accuracy of 82%[61]. Metabolomic markers will require further research to determine their potential value in the early detection of PDAC.

Core fucosylation (CF) is a form of glycosylation mediating post-translational modification of cellular protein. CF-glycosylation of an isoform of α-fetoprotein (AFP-L3) is a sensitive biomarker of hepatocellular carcinoma (HCC). Tan *et al*[62] recently published an elegant study describing differentially expressed CF peptides distinguishing between serum of healthy control and patients with pancreatic cancer. Research in this field is at a nascent stage and carries great future promise. Another approach that has recently emerged as an exciting new avenue for biomarker discovery in PDAC is the detection of discriminatory cytokine biomarker panels[63].

These and other novel molecular approaches on the discovery horizon warrant further study in the search for better methods of to improve early detection of PDAC.

CONCLUSION

The number of promising biomarkers for early diagnosis of pancreatic cancer has risen dramatically in recent years. However despite the large number of studies and new discoveries, no molecular approach has been rigorously validated or ascended to application for routine clinical use to date. Mutational analysis points to the heterogeneity in acquired genetic changes in PDAC and underscores the complexity and obstacles inherent to the use of mutations as candidate markers for detection. Other classes of markers, such as aberrantly methylated DNA or miRNA, may be more informative and practical at this time. Carefully designed clinical validation studies are now needed to sort out which molecular markers measured, which assay platforms, and in which biological media will prove to be of greatest value in screening and surveillance applications.

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TOPIC HIGHLIGHT

2015 Advances in Pancreatic cancer

Cancer immunotherapy for pancreatic cancer utilizing α-gal epitope/natural anti-Gal antibody reaction

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) has the poorest prognosis of all malignancies and is largely resistant to standard therapy. Novel treatments against PDAC are desperately needed. Anti-Gal is the most abundant natural antibody in humans, comprising about 1% of immunoglobulins and is also naturally produced in apes and Old World monkeys. The anti-Gal ligand is a carbohydrate antigen called "α-gal epitopes" with the structure Galα1-3Galβ1-4GlcNAc-R. These epitopes are expressed as major carbohydrate antigens in non-primate mammals, prosimians, and New World monkeys. Anti-Gal is exploited in cancer vaccines to increase the immunogenicity of antigen-presenting cells (APCs). Cancer cells or PDAC tumor lysates are processed to express α-gal epitopes. Vaccination with these components results in *in vivo* opsonization by anti-Gal IgG in PDAC patients. The Fc portion of the vaccine-bound anti-Gal interacts with Fcγ receptors of APCs, inducing uptake of the vaccine components,

transport of the vaccine tumor membranes to draining lymph nodes, and processing and presentation of tumor-associated antigens (TAAs). Cancer vaccines expressing α-gal epitopes elicit strong antibody production against multiple TAAs contained in PDAC cells and induce activation of multiple tumor-specific T cells. Here, we review new areas of clinical importance related to the α-gal epitope/anti-Gal antibody reaction and the advantages in immunotherapy against PDAC.

Key words: Pancreatic cancer; Immunotherapy; Cancer antigen; MUC1; α-gal epitopes; Cancer vaccine; Cancer stem cell; Carbohydrate research

Core tip: The goal of cancer immunotherapy is to elicit an immune response against autologous tumors and to induce multiple T cell clones against multiple tumor-associated antigens. To establish effective, next-generation immunotherapy toward pancreatic ductal adenocarcinoma (PDAC), we focus on the strong interaction between the natural human antibody, anti-Gal, and carbohydrate antigens called "α-gal epitopes". Here, we review the literature on the distribution of natural anti-Gal antibody and its ligand in mammals and characterization of the immunosuppressive microenvironment of PDAC tumors, which is a major obstacle against effective clinical immunotherapies. We also discuss immunotherapeutic strategies using the α-gal epitope/anti-Gal antibody reaction.

Tanemura M, Miyoshi E, Nagano H, Eguchi H, Matsunami K, Taniyama K, Hatanaka N, Akamatsu H, Mori M, Doki Y. Cancer immunotherapy for pancreatic cancer utilizing α-gal epitope/natural anti-Gal antibody reaction. *World J Gastroenterol* 2015; 21(40): 11396-11410 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i40/11396.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i40.11396

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is common worldwide, and its incidence is gradually increasing in the United States, with an estimated 43920 new cases and 37390 deaths in 2012[1]. Surgical resection is the only known curative treatment for PDAC[2], and patients who develop a recurrence usually present with the recurrence between 9 and 12 mo after resection[3]. The median survival of PDAC patients following surgery is 15-20 mo, with a 5-year survival rate of approximately 20%[3,4]. Accordingly, the median survival of patients with locally advanced, unresectable PDAC is very poor[2,5]. Currently, only a few chemotherapeutic agents have been shown to be effective against PDAC, including gemcitabine and a combination of fluorouracil, leucovorin, oxaliplatin, and irinotecan, which is called the FOLFIRINOX regimen[6,7]. Unfortunately, the survival of patients treated with these regimens is marginal.

From the point of view of the PDAC microenvironment, reciprocal interactions between cancer cells and host cells including fibroblasts and inflammatory and vascular endothelial cells orchestrate a micro-environment that is immunosuppressive, fibrotic, and poorly vascular[8-10]. This desmoplastic reaction that surrounds PDAC lesions constitutes a major obstacle to the efficacy of therapy[11]. Indeed, cytotoxic drugs poorly penetrate this dense stromal matrix. Hence, novel therapeutic approaches against PDAC are urgently needed. As immunotherapies act differently than conventional therapies, including chemotherapy or radiation therapy, they are a promising alternative treatment modality for this deadly disease.

Here, we review relevant immunotherapies and address the basic problems with cancer immunotherapy. We detail our recent strategy for vaccination with tumor antigens exploiting the interaction between α-gal epitopes and anti-Gal antibody. The ligand for anti-Gal is a carbohydrate antigen called α-gal epitope with the structure Galα1-3Galβ1-4GlcNAc-R, which is on carbohydrate chains of glycolipids and glycoproteins[12]. Furthermore, we also discuss our novel immunotherapy approach that targets pancreatic cancer stem cells (CSCs) using stem cell markers that are engineered to express α-gal epitopes.

Epidemiology and clinical management of pancreatic cancer

PDAC is the fifth leading cause of cancer-related death in the developed world, with more than 260000 deaths annually worldwide[13]. Surgical resection (resectable disease) is the only curative treatment. However, the 5-year survival rate after surgical resection is only 5.5%-21%[2]. Radiation therapy (as combined modality therapy for locally advanced/unresectable disease) and chemotherapy (as adjuvant treatment for both locally advanced/unresectable and metastatic disease) have become a part of the armamentarium of therapy for PDAC[14,15]. However, most patients present with advanced, unresectable disease, and even those that undergo successful surgical resection have high recurrence rates, with an average overall survival of 16-18 mo[14,15]. Chemotherapeutic options include gemcitabine-based therapy[16] and more recently, FOLFIRINOX in select patients with a favorable performance status[6,7]. We and others have reported encouraging survival rates following preoperative gemcitabine-based chemoradiotherapy in patients with potentially resectable PDAC[17-19]. Despite modest improvements in mortality and quality of life, the benefits of treatment remain limited, and cures are rare.

The poor prognosis of PDAC is related to a combination of late detection and relatively ineffective

standards of care. Several promising drugs that target important characteristics of malignancy, such as angiogenesis, proliferation, and metastasis, have failed to provide clinically relevant benefits and have provided only trivial improvements in disease-free survival and overall survival rates.

As immunotherapies act differently than standard treatments (chemotherapy and radiation therapy), they represent a promising alterative treatment modality for this deadly disease. Immunotherapies use techniques such as vaccination that is designed to activate the patient's immune system with tumor-associated antigens (TAAs) expressed in PDAC cells. The immune system that has been activated by vaccination can recognize TAAs and eradicate cancer cells. Although several clinical studies have documented evidence of treatment-induced, antigen-specific immune responses, few, if any, protective immune responses have been observed in patients with metastatic disease[20]. In addition, vaccination against TAAs is an attractive approach as an adjuvant-setting treatment after surgery when tumor-induced immune suppression is minimal[21,22]. Effective anticancer functions of the immune system require cytotoxic $CD8^+$ T cells, Tn helper-1 (Th-1) cells, mature dendritic cells (DCs), activated pro-inflammatory macrophages (M1), and natural killer cells. However, PDAC cells induce both local and systemic immune dysfunction, thus avoiding detection by the immune system[8-10,23].

Immune cells in PDAC promote an immunosuppressive, anti-inflammatory environment, which is a major obstacle in clinical immunotherapy

At the level of cancer cells, PDAC cells induce both local and systemic immune dysfunction via at least three mechanisms involving modulation of the immune system and avoidance of detection by effector cells: (1) contact-dependent factors [*i.e.*, expression of immune system checkpoint ligands such as ligand for programmed death-1 (PD-L1)]; (2) secretion of soluble immunosuppressive factors such as interleukin (IL)-10, transforming growth factor (TGF)-β, and vascular endothelial growth factor; and (3) interference with major histocompatibility complex (MHC) class Ⅰ peptide presentation by downregulation of MHC class Ⅰ expression, disabling antigen degradation, or preventing antigen insertion into the MHC class Ⅰ groove (Figure 1).

The tumor microenvironment of PDAC consists of not only cancer cells but also immune suppressive cells such as cancer-associated fibroblasts (CAFs), tolerogenic DCs, myeloid-derived suppressor cells (MDSCs), immunosuppressive tumor-associated macrophages (TAMs), and regulatory T cells (Tregs) (Figure 1). These immunosuppressive cells in PDAC can inhibit the anti-tumor immunity that is induced by vaccines. Accumulation of these immunosuppressive cells may be closely related to the extent of disease and may contribute to the failure to provide clinically relevant benefits. CAFs secrete fibroblast activation protein (FAP-α), which further suppresses effector T cells by interfering with tumor necrosis factor- and interferon-γ-related activation[24,25]. FAP-α is overexpressed in both the PDAC stroma and on PDAC cells[26], and anti-FAP-α monoclonal antibodies are currently in clinical development. MDSCs are immature myeloid cells that suppress both innate and adoptive immunity[27]. Factors contributing to their action in immunity include sequestration of cysteine, expression of high levels of arginase, impairment of T cell homing to lymph nodes, and secretion of TGF-β. These factors inhibit the function of effector T cells and natural killer cells and promote the development of Tregs. Patients with PDAC have increased numbers of MDSCs in their circulation compared to healthy controls, and MDSC numbers are correlated with levels of the Th-2 cytokine IL-13 and Treg cell numbers[28,29]. An increased number of circulating MDSCs is an independent poor prognostic factor in PDAC patients[28,29]. Furthermore, TAMs interact with the immune system *via* multiple mechanisms such as secretion of IL-10 and TGF-β and expression of immune inhibitory ligands such as PD-L1. In PDAC, TAMs are significantly increased in tumor tissue[30,31]. Patients with PDAC have increased numbers of Tregs, both in the circulation and in tumor tissues. By expression of cytotoxic T lymphocyte antigen-4 and secretion of IL-10 and TGF-β, Tregs suppress the exaggerated immune responses induced by vaccination[32,33]. Conversely, a low Treg percentage in the circulation 1 year after surgical resection is correlated with improved survival[34]. Taken together, these cellular subtypes, including CAFs, MDSCs, TAMs, and Tregs, are potent obstacles against effective clinical immunotherapies.

Reciprocal distribution of the natural anti-Gal antibody and its ligand, α-gal epitopes, in mammals

Anti-Gal is the most abundant antibody in humans, comprising about 1% of immunoglobulins, and is present as IgG, IgM, and IgA isotypes[35,36]. Anti-Gal is continuously produced throughout life as an immunological response to antigenic stimulation by bacteria of the normal flora, including *Klebsiella pneumonia*, *Escherichia coli*, and *Serratiamarcecens*[35,36]. As many as 1% of human B cells are capable of producing anti-Gal[12], most of which are quiescent; only those in the gastrointestinal tract produce this antibody in response to continuous antigenic stimulation by gastrointestinal bacteria. Anti-Gal in humans is encoded by several heavy-chain genes, primarily of the VH3 immunoglobulin gene family[12,37]. The distribution of anti-Gal in mammals is unique (Figure 2). Anti-Gal is produced only in humans and Old World primates (monkeys of Asia and Africa). In contrast, all other mammals including non-primate

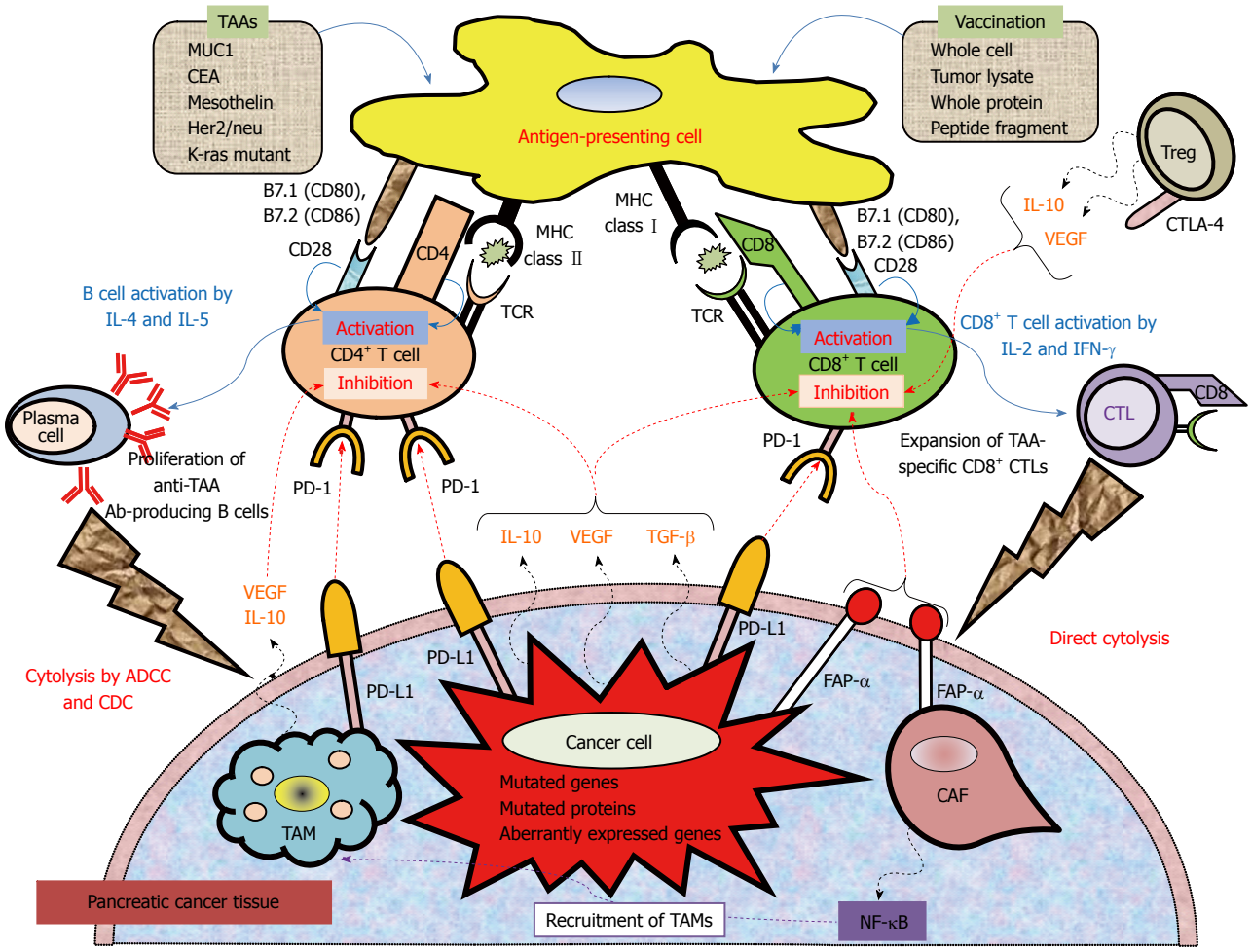

Figure 1 Mechanistic basis for vaccine-based immunotherapy, and immune co-stimulatory, co-inhibitory ligands and receptors, and soluble immune modulating factors involved in T cell activation and inhibition. Anticancer immunotherapy aims to harness the natural ability of the immune system to recognize and react against potential TAAs. Peptide-based, protein-based, or whole cell-based vaccines rely on identified immune-dominant TAA epitopes to stimulate anticancer T-cell responses. Antigen-presenting cells (APCs), including dendritic cells, capture antigens obtained from vaccinations. After intracellular processing, antigen peptides are loaded onto major histocompatibility complex (MHC class Ⅰ or Ⅱ) molecules on the surface of the APC. Specific T cells encounter these MHC-peptide complexes in conjugation with a co-stimulatory signal. The activated T cells proliferate and secrete cytokines (IL-2, IL-4, IL-5, INF-γ), resulting in the production of a cascade of immune effector cells. Immunotherapeutic strategies that inhibit immune checkpoints such as those mediated by CTLA-4 and PD-1 reduce the barriers that vaccines must overcome to trigger therapeutically relevant anticancer immune responses. Recently, preclinical and clinical studies have demonstrated that combining immune-modulating agents such as cyclophosphamide (CY) and checkpoint inhibitors (anti-CTLA-4, anti-PD-1, anti-PD-L1) with vaccine strategies can enhance anticancer immune responses as well as block the tolerizing mechanisms that would otherwise inhibit these responses. ADCC: Antibody-dependent cell-mediated cytotoxicity; CAF: Cancer-associated fibroblast; CDC: Complement-dependent cytolysis; CTLA-4: Cytotoxic T lymphocyte antigen-4; FAP-α: Fibroblast activation protein-α; TAM: Tumor-associated macrophage; TCR: T cell receptor; PD-1: Programmed cell death protein 1; PD-L1: Programmed death-ligand.

mammals (*e.g.*, kangaroos, mice, rats, pigs, dogs, horses, lions, and dolphins), prosimians (*e.g.*, lemurs), and New World monkeys (monkeys of central and south America) produce only the specific ligand for anti-Gal and not the antibody (Figure 2). The ligand for anti-Gal is a carbohydrate antigen called the α-gal epitope with the structure Galα1-3Galβ1-4GlcNAc-R, which is present on carbohydrate chains of glycolipids and glycoproteins[12]. In 1968, Eto *et al*[38] were the first to isolate the glycolipid ceramidepentahexoside, which contains the non-reducing terminal sequence Galα1-3Galβ1-4GlcNAc-R, from rabbit red blood cells (RBCs). Subsequently, the structure of rabbit RBC ceramidepentahexoside was further characterized by Stellner *et al*[39] in 1973. The synthesis of α-gal epitopes in mammals is catalyzed by the glycosylation enzyme α1, 3 galactosyltransferase (α1, 3 GT). As α-gal epitopes are abundant in both marsupials and placental mammals and absent in non-mammalian vertebrates (fish, reptiles, and birds), the α1, 3 GT gene and α-gal epitope appeared in mammalian evolution at least 140 million years ago (Figure 2). The continued prevention of anti-Gal production in mammals by natural selection throughout this evolutionary period may have resulted in the elimination of anti-Gal-encoding immunoglobulin genes from the mammalian genome[12].

The only known exceptions to anti-Gal production in mammals are in Old World monkeys, apes, and humans[12], which all have an inactive α1, 3 GT gene as the result of a few single-base deletions that generate premature stop codons that truncate the enzyme molecule, resulting in an inactive protein[12].

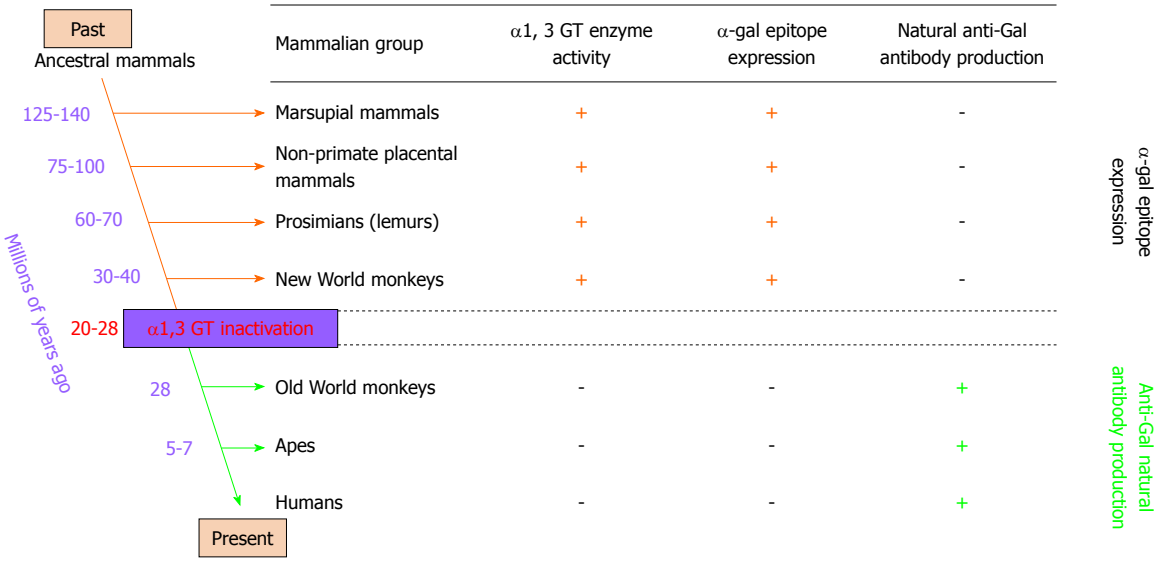

Figure 2 Reciprocal evolution of α1, 3 galactosyltransferase (α1, 3 GT) enzyme activity, α-gal epitopes, and anti-Gal antibody in mammals. α-gal epitopes have been synthesized in mammals by α1, 3 galactosyltransferase (α1, 3 GT) for more than 125 million years, since before the divergence of placental mammals and marsupials. All non-mammalian vertebrates lack α1, 3 GT and do not express α-gal epitopes. Expression of this epitope was suppressed in ancestral Old World primates after they diverged from New World monkeys, and probably after apes and monkeys diverged from each other. Suppression of α-gal epitopes was followed by production of natural anti-Gal antibody, which is absent in non-primate mammals, prosimians, and New World monkeys.

Based on the sequence of the α1, 3 GT pseudogene in Old World primates and humans, inactivation of the α1, 3 GT gene in ancestral Old World primates may have occurred 20-28 million years ago[12] (Figure 2), and the inactivation may have been associated with a major catastrophic epidemiological event that affected only ancestral Old World primates[12]. New World monkeys and lemurs were not subjected to this selective pressure because they evolved in geographical areas that were separated from the Old World land mass by oceanic barriers. Primates with an inactivated α1, 3 GT gene lack the α-gal epitope and thus are not immunotolerant to it. The anti-Gal antibody, if produced following inactivation of the α1, 3 GT gene, may provide immune protection to ancestral Old World primates against pathogens endemic to the Old World land mass that were detrimental to primates that expressed α-gal epitopes[12]. Several pathogens, including enveloped viruses[12], bacteria[12], and protozoa[12], express α-gal epitopes and can be destroyed by anti-Gal binding.

Anti-Gal antibody interacts specifically with α-gal epitopes on glycolipids and glycoproteins. Anti-Gal was initially discovered on RBCs of patients with β-thalassemia, on normal human senescent RBCs[12,40], and on sickle cell anemia RBCs. A cryptic antigen capable of binding anti-Gal may be present on human RBCs that are about 120 d old or on thalassemia and sickle cell anemia RBCs on which this antigen is present on younger RBCs[12,40]. The amount of this cryptic antigen on RBCs is very low, resulting in markedly high binding of anti-Gal, which is detrimental[41].

Although anti-Gal contributes to a number of pathological phenomena, this antibody is ubiquitous in humans. Furthermore, anti-Gal activity is found in cancer patients with solid tumors, including colon cancer, ovarian cancer, and PDAC and in patients with B cell lymphoma; anti-Gal activity is similar in patients with various types of cancer and healthy individuals[40]. Anti-Gal may be amenable to exploitation in a number of clinical settings such as cancer immunotherapy, as described in this review.

Interaction of anti-Gal/α-gal epitopes as a barrier in clinical xenotransplantation

Xenotransplantation, or transplantation of organs and tissues from animals such as pigs into humans, is of considerable clinical importance because the number of human organ donors is insufficient[42,43]. Pigs are considered to be the most suitable organ donors because their organs are similar in size and function as many human organs[42,43]. However, pig cells express very high levels of α-gal epitopes[35]. Anti-Gal in xenograft recipients binds to α-gal epitopes on the endothelial cells of xenografts and induces complement-dependent cytolysis followed by platelet aggregation, occlusion of small blood vessels, collapse of the vascular bed, and hyperacute rejection of the xenograft within 0.5-24 h (Figure 3)[35]. An additional complicating factor in xenotransplantation is associated with the important finding that approximately 1% of B cells in humans produce anti-Gal[35,36]. When a xenograft is transplanted into humans, the released α-gal glycoproteins activate these quiescent B cells to produce anti-Gal. The anti-Gal IgG titer increases by approximately 100-fold due to increases in both the concentration and affinity of the antibody[41]. In noteworthy studies performed by Groth and Galili,

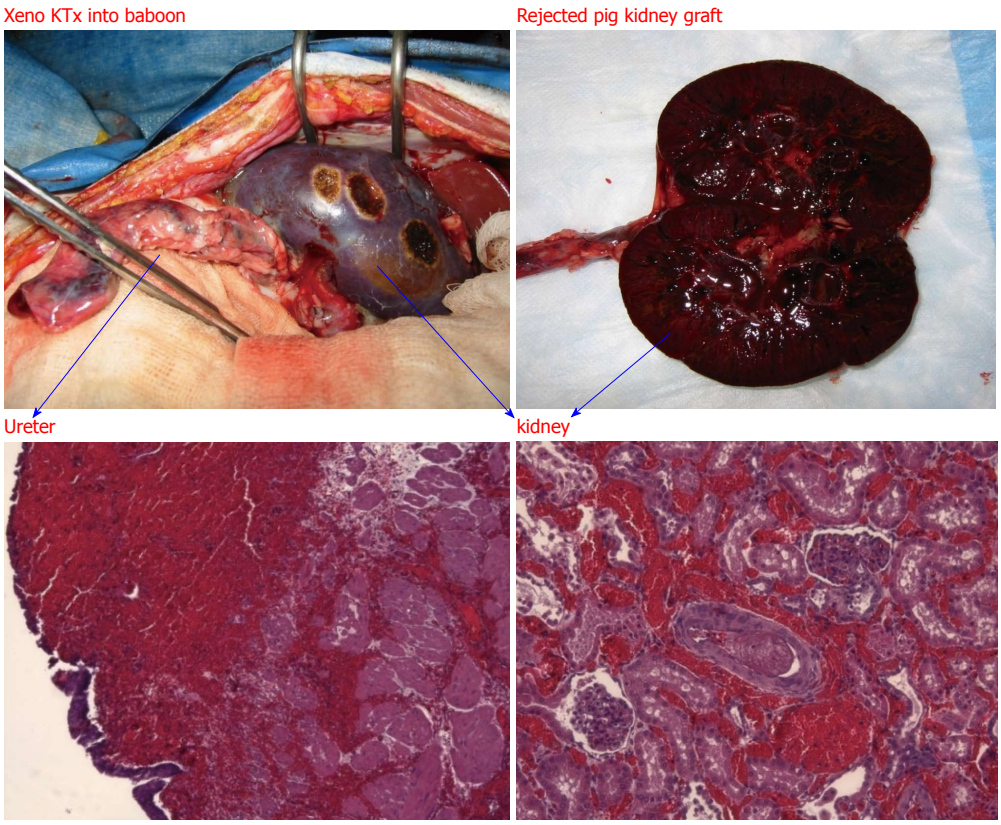

Figure 3 Hyperacute rejection of α-gal$^{+/+}$ pig kidney xenografted into a baboon (1 d after kidney transplantation). The interaction of natural baboon anti-Gal antibody with millions of α-gal epitopes expressed on the pig cell surface causes strong xenograft rejection. The *in vivo* binding of anti-Gal antibody to α-gal epitopes on transplanted pig heart or kidney is the main cause of hyperacute rejection of such grafts in humans and Old World monkeys. The recent generation of α1, 3 GT knockout pigs that lack α-gal epitopes has resulted in the elimination of this immunological barrier.

they clearly demonstrated such an increase in anti-Gal activity in patients with diabetes who received both an allogeneic kidney and fetal pig islets. This increase in anti-Gal occurred despite immunosuppressive treatment that was potent enough to prevent rejection of the kidney allograft[40,44]. This elicited anti-Gal IgG activity is likely to mediate destruction of xenograft cells by antibody-dependent cell-mediated cytotoxicity.

Fortunately, this immunological barrier was overcome in 2003 by the generation of α1, 3 GT knockout pigs, which lack α-gal epitopes[45,46]. Accordingly, heart and kidney xenografts from these knockout pigs transplanted into monkeys survived for several months[42,46-48]. The detrimental anti-Gal/α-gal epitope interaction that occurs in xenotransplantation may be harnessed for beneficial purposes in other clinical areas such as immunotherapy.

Principals of PDAC treatment with immunotherapy

Because currently available therapies have significant limitations, PDAC is an ideal setting for the development of novel treatment modalities such as immunotherapy. However, certain obstacles must be overcome for immunotherapeutic regimens against PDAC to be successful.

Tumor cell vaccines have been considered for use in immunotherapy. The simplest vaccine approach that has been applied in PDAC is inoculation of individuals with irradiated tumor cells (*i.e.*, whole cancer cell-based vaccines). This approach has the following advantages[49-51]. Whole cancer cell-based vaccines circumvent the need for targeting a selected TAA as they rely on irradiated tumor cells that by definition express a panel of TAAs. In this setting, allogeneic preparations overcome the technical difficulties that may be posed by the production of autologous vaccines, which require the isolation of a sufficient amount of malignant tissue from patients. Whole cell-based vaccines also provide non-biased immunization of lymphocytes and sera against TAAs, resulting in the generation of a reagent that may be used to identify immunologically relevant TAAs for use in the design of antigen-specific vaccination strategies.

In general, cytotoxic T cell lymphocytes play a critical role in the immunological cascade that ultimately results in the lysis of cancer cells in a TAA-specific manner[23]. Receptors on the surface of T cells bind to TAAs or peptide fragments that are bound to MHC class I molecules, which are present on the surface of professional antigen-presenting cells (APCs) such as DCs and macrophages. T cell activation, however, also requires the presence of costimulatory molecules (*e.g.*, B7.1, B7.2), which can be provided only by professional APCs[52]. The interaction of the T

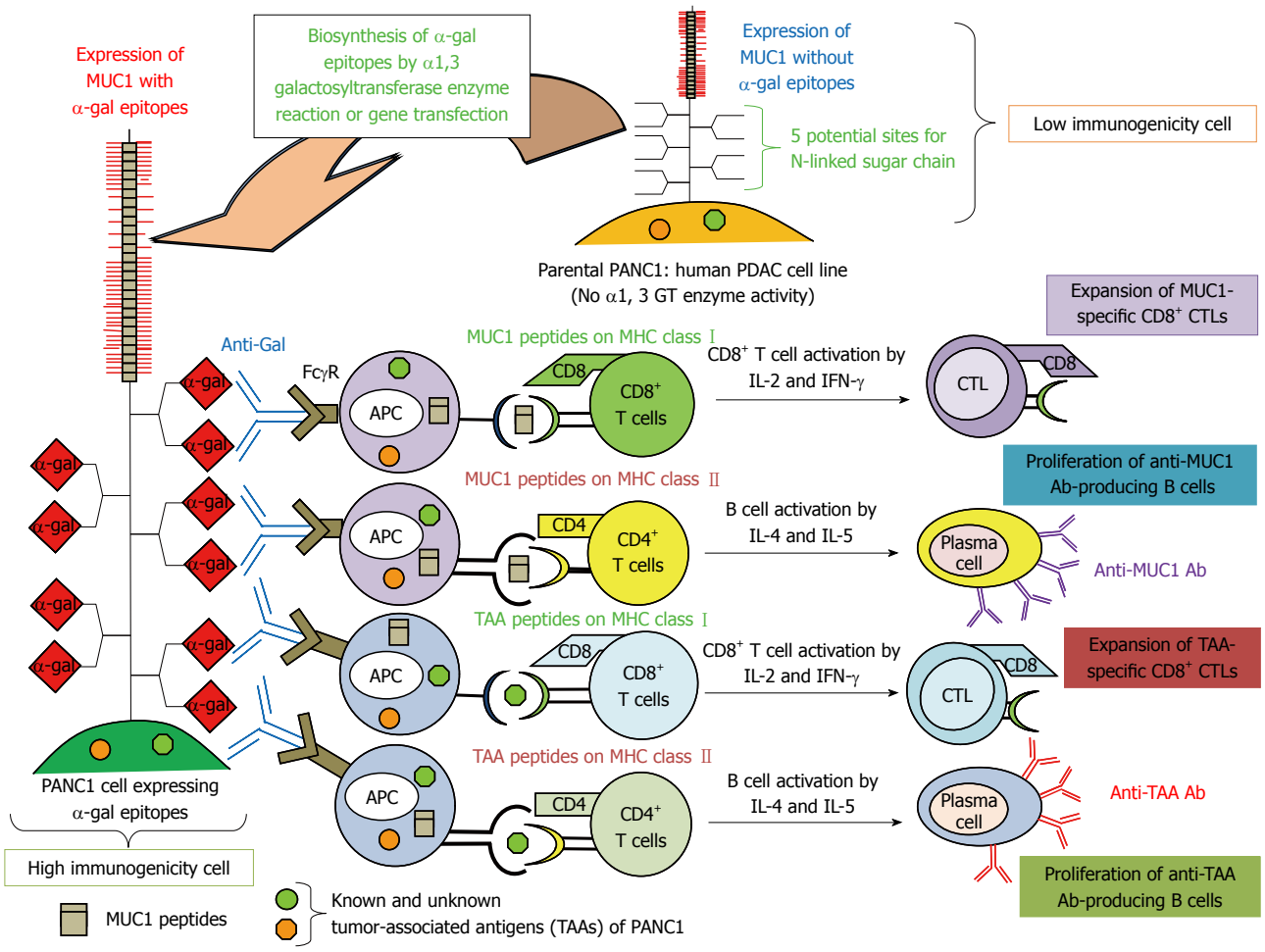

Figure 4 Increased immunogenicity of known and unknown tumor-associated antigens and MUC1 engineered to express α-gal epitopes. Immunity towards known and unknown tumor-associated antigens (TAAs), including MUC1, in PDAC patients is relatively weak, and presentation of these TAAs to the immune system is poor due low immunogenicity. We tested the effects of vaccination using immunogenetically enhanced known and unknown TAAs and MUC1 with expression of α-gal epitopes on production of antibodies for MUC1 and other TAAs derived from PDAC cells, as well as induction of tumor-specific T cell activation.

cell receptor on naïve T cells with the TAA on tumors without the delivery of a costimulatory nonspecific signal (Signal 2) is thought to result in the T cell entering a state of long-term unresponsiveness to the TAA, called anergy[53-55]. Once T cells are activated, helper T cells are recruited that secrete cytokines such as IL-2 and granulocyte macrophage colony-stimulating factor, which further enhances T cell activation and proliferation (Figure 1). Accordingly, T cells require these two signals to become fully activated[56]. Despite these immunological responses to the presence of PDAC cells, effective immunity does not develop against PDAC cells because of impaired tumor recognition by immune cells, poor immunogenicity of TAAs, and the presence of an immunosuppressive milieu in the PDAC tumor microenvironment, which includes CAFs, MDSCs, TAMs, and Tregs (Figure 1).

Another reason for the absolute requirement for effective uptake of whole cell-based vaccines by APCs is that activation of TAA-specific T cells does not occur at the vaccination site, but rather takes place within the draining lymph nodes of the vaccination sites or in the spleen. Only after they are activated can tumor-specific T cells leave the lymph nodes or spleen to seek and destroy cancer cells that express the TAAs. For such activation to occur, the whole cell-based vaccine must be transported from the vaccination site by APCs to lymph nodes or the spleen[57,58]. Transportation of vaccines occurs only after effective uptake of the vaccine by APCs[57,58].

Improving APC targeting through formation of immune complexes containing α-gal epitopes and anti-Gal

As described above, TAA molecules expressed on whole cell-based vaccines are not modified to express markers that allow effective recognition by APCs. This section describes how whole cell-based vaccines can be directed to APCs through formation of immune complexes that interact with Fcγ receptors (FcγRs) on APCs. The carbohydrate make-up of whole cell-based vaccines can be modified to include expression of α-gal epitopes (Figure 4). These epitopes are recognized by naturally abundant anti-Gal antibodies that opsonize the whole cell-based vaccines, and the resulting immune

complex enhances the immunogenicity of the whole cell-based vaccine. APCs, including macrophages, skin Langerhans cells, and blood-derived DCs, all express FcγRs (*e.g.*, Fcγ RI/CD64, Fcγ RⅡ/CD32, Fcγ RⅢ/CD66). These FcγRs bind and mediate the internalization of opsonized cells (*i.e.*, cells with bound IgG molecules), cell membranes, or molecules (all defined as cancer antigens) *via* the Fc portion of the opsonizing IgG antibody[59-61]. This results in enhancement of the immunogenicity of the antigen that is complexed with an IgG antibody. Thus, vaccination of cancer patients with a tumor cell vaccine that is modified to express α-gal epitopes should result in *in situ* binding of the patient's anti-Gal IgG molecules to α-gal epitopes on the vaccinating cell membrane. This targets the vaccines to APCs by interaction of the Fc portion of the anti-Gal antibody on the vaccinating cell membrane with FcγRs on the APCs[62,63]. This interaction induces the uptake of the whole cell-based vaccine by APCs, which subsequently transport the vaccinating tumor membranes to the draining lymph nodes or spleen.

In our previous study[64], we investigated the beneficial effects of whole cell-based vaccines with α-gal epitope-expressing pancreatic cancer cells in the induction of tumor-specific B- and T-cell responses, *in vivo* prevention of tumor growth, and improvement in survival[64]. We employed a human pancreatic cell line, PANC1, which endogenously expresses Mucin1 (MUC1) in the whole cell-based vaccine. MUC1 can be used as a tumor marker and is a potential target for PDAC immunotherapy. However, vaccination with MUC1 peptides fails to stimulate an immune response against PDAC because immunity toward TAAs, including MUC1, in PDAC patients is relatively weak, and the presentation of these TAAs to the immune system is poor due to their low immunogenicity (Figure 4). To increase the immunogenicity of the PANC1 whole cell-based vaccine, which includes unknown TAAs and the MUC1 antigen against APCs, we modified these cells to express α-gal epitopes by transfection of the mouse α1, 3 GT gene (designated here as α-gal PANC1) (Figure 4). This modified whole cell-based vaccine takes advantage of anti-Gal antibodies, resulting in increased uptake of TAAs contained in the tumor cell vaccine in an antibody-dependent manner. Simultaneously, MUC1 can also be engineered to express α-gal epitopes, because the MUC1 molecule has five potential sites for N-glycans and can bind anti-Gal *in situ* at the vaccination sites (Figure 4).

In Figure 5A, we show a schematic illustration of an experimental protocol. The anti-Gal antibody as a natural antibody is not present in naïve α1, 3 GT knockout mice. Repeated immunizations with pig kidney fragments result in the appearance of anti-Gal antibodies, with an anti-Gal IgG titer that is similar to that observed in a large proportion of samples of human serum. *In vitro* analysis of the immune response showed that three vaccinations with α-gal PANC1 elicited a strong anti-MUC1 IgG response, whereas vaccination with whole parental PANC1 cells did not elicit such an antibody response (Figure 5B). Furthermore, α-gal PANC1 whole cell-based vaccines induced a protective immune response against a tumor challenge with the MUC1-expressing B16F10 melanoma cell line (Figure 5C). The beneficial effects of α-gal PANC1 whole cell-based vaccines are illustrated by prolonged survival after tumor challenge.

PDAC tumor lysates that are engineered to express α-gal epitopes can target pancreatic CSCs

In previous sections, we described the *in vitro* and *in vivo* effects of whole cell-based vaccination with α-gal epitope-expressing pancreatic cancer cells[64]. However, the effect was somewhat weak as shown in Figure 5C. To further develop an effective immunotherapy for PDAC, we hypothesized that the tumor lysate is a more suitable source of TAAs for vaccination because it contains several known and unknown antigens expressed in cancer cells and stromal cells that can elicit a broad-spectrum anti-tumor immune response (Figure 6). Moreover, the primary PDAC tumor tissue contains a subset of putative pancreatic CSCs[65-69] (Figure 6). These pancreatic CSCs are resistant to the standard cytotoxic agent gemcitabine and show enhanced metastatic potential[65-69]. Additionally, inducing an immune response against pancreatic CSCs, which constitute only 1% of all cancer cells, is often difficult[65-69].

In the newest study in our institute, we prepared a polyvalent tumor lysate vaccine that was engineered to express α-gal epitopes on primary PDAC tumors (designated α-gal tumor lysate vaccine)[70]. Accordingly, α-gal tumor lysate vaccines should be able to increase the immunogenicity of the broad spectrum of TAAs, which are present in differentiated pancreatic cancer cells, pancreatic CSCs, and stromal cells (Figure 6). As shown in Figures 7 and 8, we investigated the beneficial effects of the α-gal tumor lysate vaccine using adoptive transfer models. The tumor growth of live PDAC cells, which include differentiated pancreatic cancer cells and pancreatic CSCs, in non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice was examined. The experimental design of the adoptive transfer model using NOD/SCID mice is shown in Figure 7. High anti-Gal knockout mice were generated as described in a previous study[41]. Subsequently, these mice were vaccinated with either the parental tumor lysate or an α-gal tumor lysate vaccine. To compare the effectiveness of the α-gal whole cell-based vaccine with that of the α-gal tumor lysate vaccine, the NOD/SCID mice were given *ip* injections of an α-gal whole cell-based vaccine consisting of 1×10^6 cells irradiated with 50 Gy in a manner similar to the tumor lysate vaccine. One week after the last vaccination, splenocytes were prepared from successfully vaccinated donor knockout

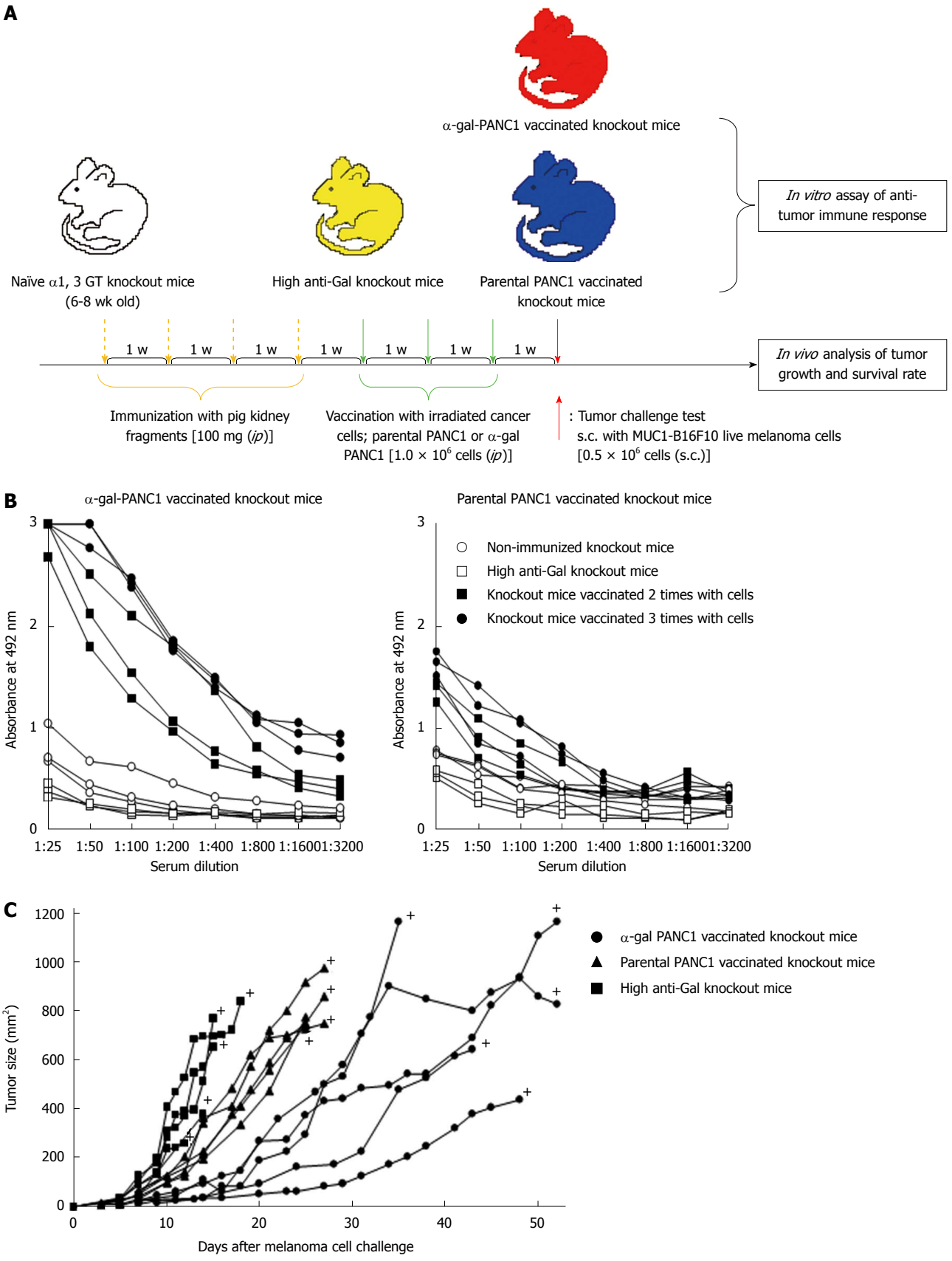

Figure 5 Experimental design for *in vitro* and *in vivo* studies and anti-MUC1 IgG antibody production assessed with an enzyme-linked immunosorbent assay. A: Schematic illustration of the experimental protocol; B: Anti-MUC1 IgG production in knockout mice vaccinated with α-gal PANC1, and anti-MUC1 IgG production in knockout mice vaccinated with parental PANC1; C: Size of subcutaneous tumors after challenge with MUC1-B16F10 cells. +: Death.

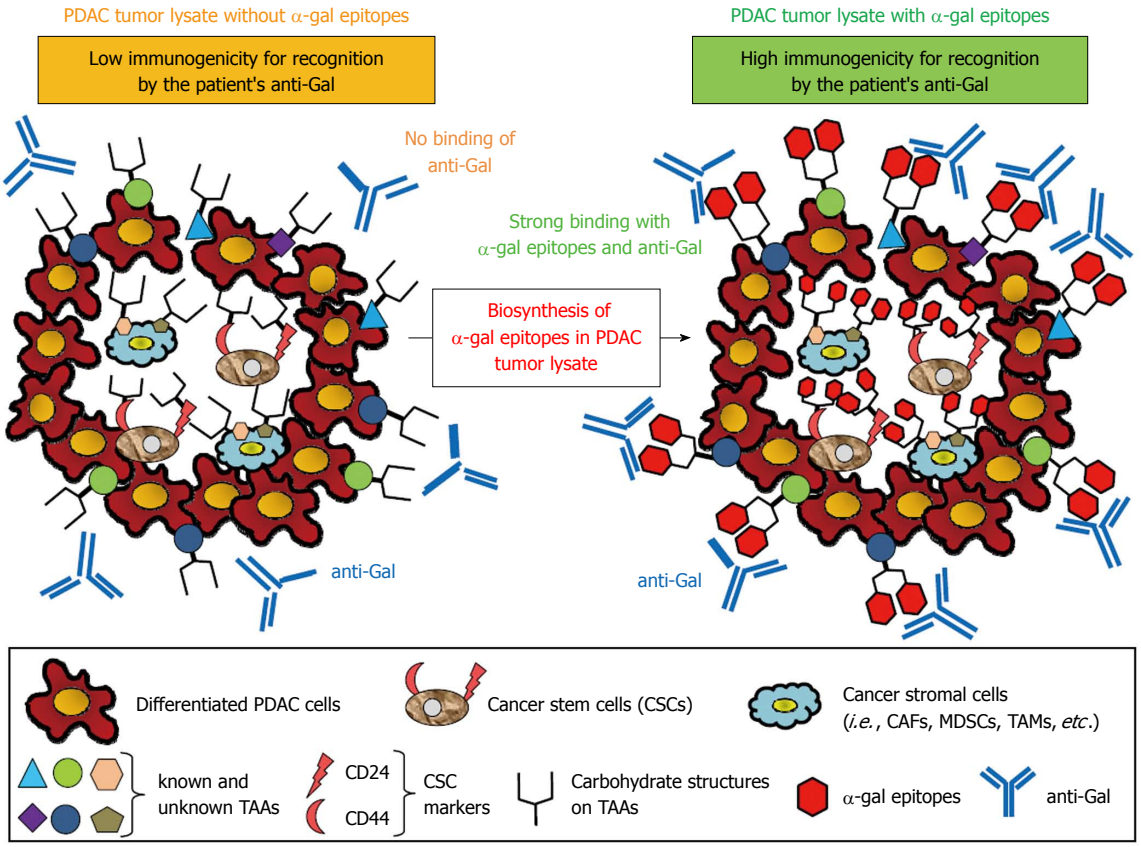

Figure 6 Concept of effective vaccination with α-gal tumor lysate against pancreatic ductal adenocarcinoma. A tumor lysate is a more suitable source of tumor-associated antigens (TAAs) because it contains several known and unknown antigens in cancer cells and stromal cells that can elicit a broad-spectrum anti-tumor immune response. Moreover, the primary tumor of pancreatic adenocarcinoma contains a subset of pancreatic cancer cells with stem cell properties (*i.e.*, pancreatic cancer stem cells: pancreatic CSCs). To increase the immunogenicity of known and unknown TAAs, CSC markers, or TAAs contained in cancer stromal cells to antigen-presenting cells, anti-Gal bound to α-gal-expressing TAAs could be a suitable strategy.

mice. For adoptive transfer, these isolated splenocytes were transferred by *ip* injection into NOD/SCID mice. One day after adoptive transfer, all NOD/SCID mice were challenged with either live PDAC cells or live pancreatic CSCs (*i.e.*, $CD44^+CD24^+$ PANC1 cells). These mice were examined for both tumor growth and survival (Figure 7). Regarding the size of subcutaneous tumors after a challenge with live PDAC cells (Figure 8A), untreated control mice, parental tumor lysate-vaccinated, and α-gal whole cell-vaccinated mice developed large tumors, whereas no tumor growth was noted in the α-gal tumor lysate-vaccinated mice[70]. Regarding the size of subcutaneous tumors after a challenge with live pancreatic CSCs, control mice, parental tumor lysate-vaccinated, and α-gal whole cell-vaccinated mice developed large tumors, but tumorigenesis by pancreatic CSCs was completely prevented in all α-gal tumor lysate-vaccinated mice (Figure 8B). With the exception of the α-gal tumor lysate group, no significant differences were found in the time to appearance of palpable tumors after tumor challenge among these three groups, including the α-gal whole-cell group. Moreover, vaccination with the parental tumor lysate and with α-gal whole-cell did not prolong the survival time after tumor challenge with pancreatic CSCs, whereas vaccination using the α-gal tumor lysate significantly improved survival after tumor challenge[70]. Taken together, *in vivo* anti-tumor effects induced by α-gal tumor lysate vaccination were markedly stronger than those with either the parental tumor lysate or α-gal whole-cell. The reason for the powerful induction of anti-tumor effects by α-gal tumor lysate vaccination was clearly shown with flow cytometry (Figure 8C). Sera from both α-gal whole-cell and α-gal tumor lysate groups more strongly bound to both $CD44^+CD24^+$ (pancreatic CSCs) and $CD44^-CD24^-$ PANC1 cells (differentiated PDAC cells) than to those from the parental tumor lysate group. Importantly, vaccination with the α-gal tumor lysate induced better antibody production against both PANC1 cell populations than α-gal whole cell-based vaccination (Figure 8C).

We conclude that the use of a tumor lysate vaccine that is engineered to express α-gal epitopes can elicit a durable and broadly protective immune response to subtypic PDAC cells, and that such vaccination may be a strategy for a universal cancer vaccine that will cure patients with PDAC.

Conclusion and future perspectives

The inability of the immune system to mount an antitumor response in PDAC despite an influx of

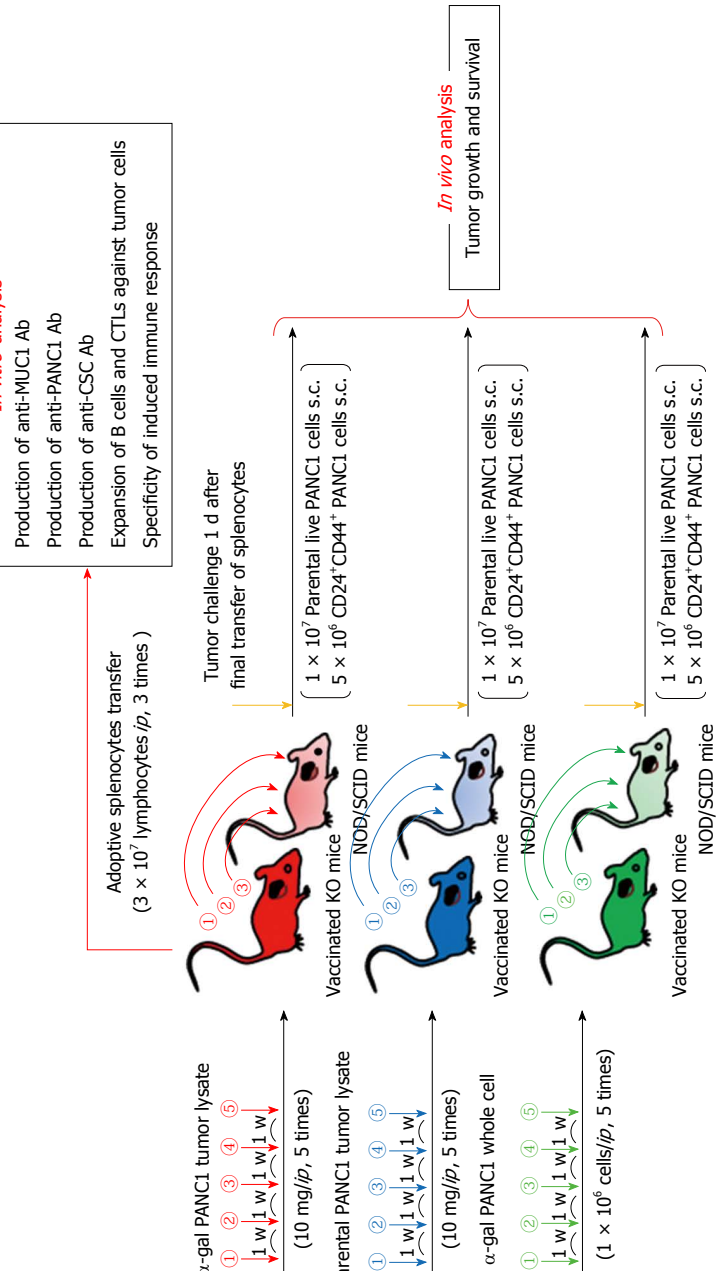

Figure 7 Experimental design of the adoptive transfer model using non-obese diabetic/severe combined immunodeficiency mice. Experimental design of *in vitro* and *in vivo* studies was shown. For *in vivo* study, the adaptive transfer model, using non-obese diabetic/severe combined immunodeficiency mice as donors was employed.

lymphocytes has led to an abundance of therapeutic approaches aiming to modify and potentiate the immune reaction. Immunotherapeutic strategies have shown defined activity in the tumor and its microenvironment. Despite an antitumor response, application of these mechanisms has not yet become a component of standard therapy, largely due to the transient, unpredictable nature of the result of treatment and the lack of advanced phase studies.

Most PDAC patients have advanced unresectable disease at the time of diagnosis. Therefore, the high tumor burden of unresectable disease may overcome the effects of immune system stimulation that is induced by immunotherapy. Moreover, genomic instability confers resistance, and a fibrotic, hypoxic, hypovascular, and immunosuppressive tumor microenvironment may limit the potential benefits of immunotherapeutic applications of mono-TAA-based immunization. Accordingly, any vaccine used in patients with PDAC should contain multiple TAAs, that is, a polyvalent tumor lysate vaccine prepared from autologous tumors. However, immunotherapy alone is limited by the number of cytotoxic T lymphocytes that can penetrate a large and established pancreatic tumor. The most encouraging results of immunotherapy in PDAC have been in adjuvant settings, such as post-surgery[21,22]. In clinical application for resectable disease, we plan to employ as vaccinating material, autologous tumor lysates prepared from surgically resected PDAC that is enzymatically processed *in vitro* to express α-gal epitopes (Figure 9). However, the tumor mass resected from the pancreas is often small and limited, and the vast majority of PDAC patients are diagnosed as inoperable because they present with incurable metastatic disease. To overcome the problem of limited tissue, we propose using tumors generated in mice for vaccination material as described in this article (Figure 9). Furthermore, the role of combination therapy with either systemic chemotherapy or radiation therapy with immunotherapy using α-gal tumor lysate

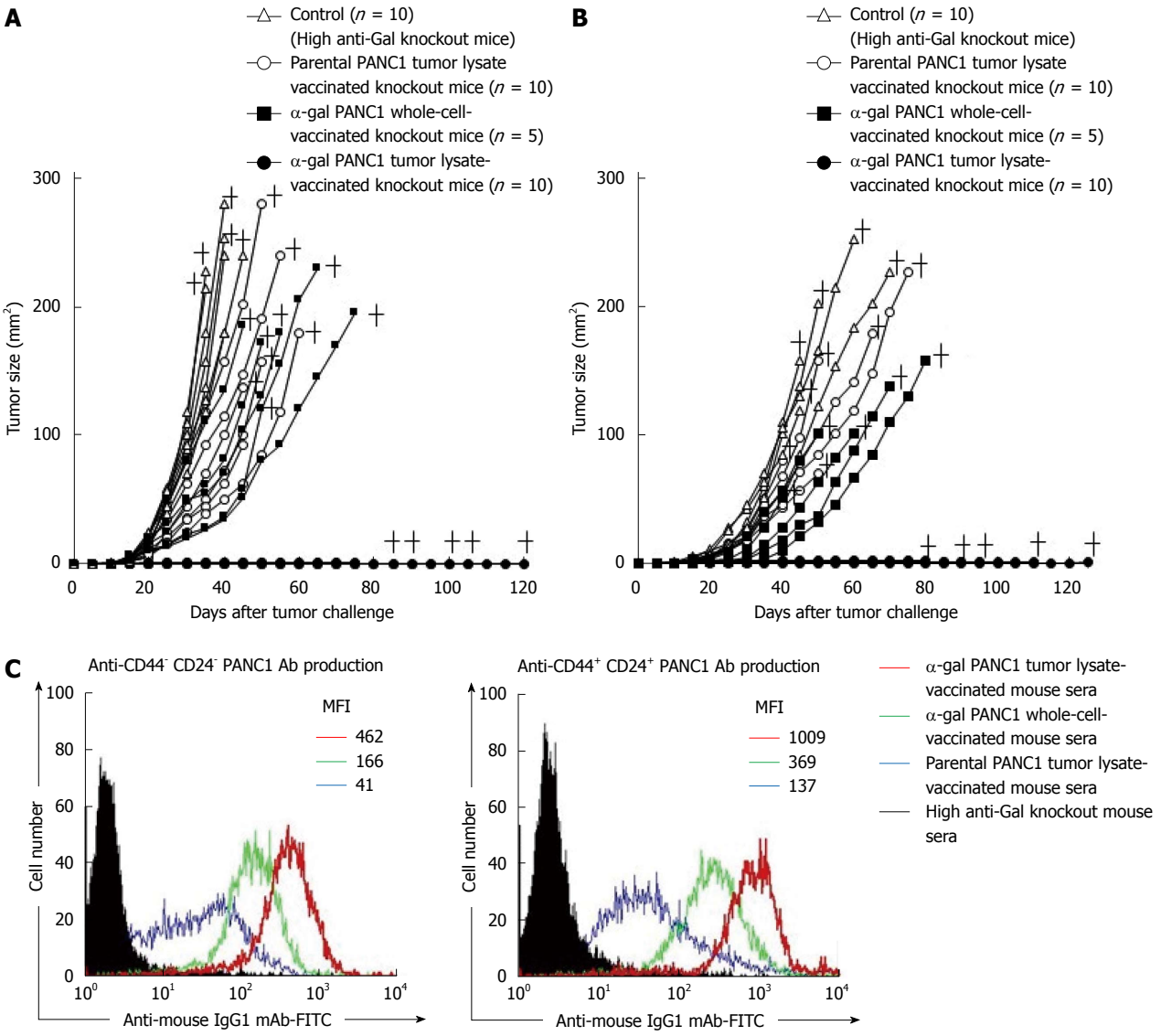

Figure 8 ***In vivo*** **tumor growth in adoptively transferred non-obese diabetic/severe combined immunodeficiency mice challenged with either live PANC1 cells or live CD44$^+$CD24$^+$ PANC1 cells, and production of antibodies against differentiated cancer cells and cancer stem cells.** A: We monitored tumor growth in splenocyte-transferred mice. No tumors were noted in the α-gal tumor lysate-vaccinated mice. No significant differences in the time to appearance of a palpable tumor after tumor challenge were observed in the untreated control group and parental tumor lysate group (untreated: 10.6 ± 2.5 d; parental tumor lysate: 11.9 ± 2.1 d). In contrast, the development of tumors in the α-gal whole cell vaccination group was significantly delayed compared with the untreated and parental tumor lysate groups (α-gal whole-cell: 16.0 ± 2.8 d, $P = 0.018$ *vs* control; $P = 0.004$ *vs* parental tumor lysate). In the untreated control group, the maximum tumor size was 100 mm^2 within 29 to 34 d (mean: 31.4 ± 2.1 d). In comparison, tumor growth to a similar size was markedly delayed in both the parental tumor lysate group (40.3 ± 6.9 d, $P = 0.007$ *vs* control) and α-gal whole-cell group (45.6 ± 8.3 d, $P = 0.0013$ *vs* control). +; death; B: The tumorigenesis of pancreatic CSCs was completely prevented in all α-gal tumor lysate-vaccinated mice. With the exception of the α-gal tumor lysate group, no significant differences were seen in the time to appearance of palpable tumors after tumor challenge among the groups (untreated: 13.1 ± 3.3 d; parental tumor lysate: 14.4 ± 3.4 d; α-gal whole-cell: 17.0 ± 3.8 d). The tumor size reached 100 mm^2 in 40.6 ± 1.8 and 48.0 ± 4.4 d in the untreated and parental tumor lysate groups, respectively. However, tumor growth to a similar size was significantly delayed in the α-gal whole-cell group (60.5 ± 7.9 d; $P < 0.001$ *vs* control; $P = 0.033$ *vs* parental tumor lysate). +; death; C: Production of either anti-CD44$^-$ CD24$^-$ PANC1 (*i.e.*, differentiated pancreatic cancer cells) antibodies or anti-CD44$^+$CD24$^+$ PANC1 (*i.e.*, pancreatic cancer stem cells) antibodies in sera of vaccinated knockout mice assessed with flow cytometry. Closed histogram; stained cells with sera from non-vaccinated knockout mice, open histogram; stained cells with sera from vaccinated knockout mice. MFI: Mean fluorescence intensity.

vaccination should also be assessed due to evidence that synergistic effects may occur when both therapies are administered simultaneously (Figure 9). We sincerely hope that the use of a tumor lysate vaccine that is engineered to express α-gal epitopes will elicit a strong immune response toward all PDAC cells, including differentiated PDAC cells and PDAC CSCs, and will improve the prognosis for patients with PDAC.

For clinical application of this effective immunotherapy, we need to assess the toxicity and safety of injection of α-gal tumor lysates in humans. Although further studies are required, we should earnestly and simultaneously engage in both clinical studies involving α-gal tumor lysate vaccination and safety studies for this novel immunotherapy against the deadly disease, PDAC.

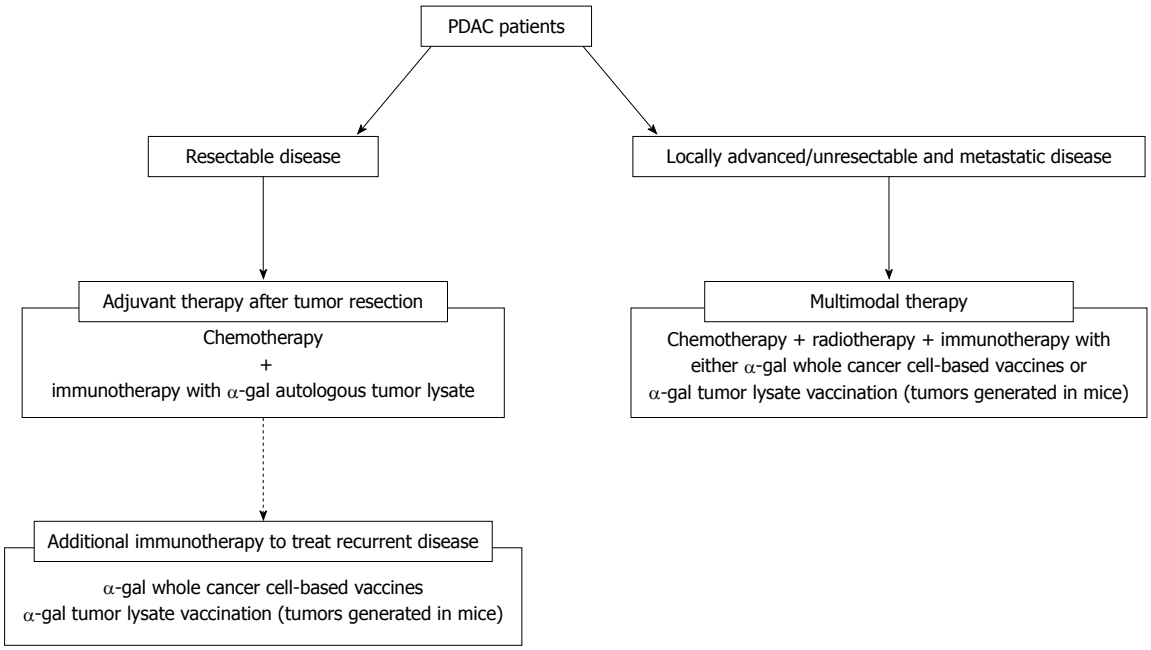

Figure 9 Treatment strategy using cancer immunotherapy utilizing α-gal epitope/anti-Gal antibody reaction for pancreatic ductal adenocarcinoma patients. The clinical implications of this cancer immunotherapy model are shown. For patients with resectable disease, we plan to employ autologous tumor lysates prepared from surgically resected PDAC that is enzymatically engineered to express α-gal epitopes. For patients with recurrent disease after surgery, additional immunotherapy with either α-gal whole cancer cell-based vaccines or α-gal tumor lysate vaccination (tumors generated in mice) should be assessed. For patients with unresectable and metastatic disease, multimodal therapy, including cancer immunotherapy using either α-gal whole cancer cell-based vaccines or α-gal tumor lysate vaccination (tumors generated in mice) should be conducted.

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REVIEW

Tight junction disruption: *Helicobacter pylori* and dysregulation of the gastric mucosal barrier

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Abstract

Long-term chronic infection with *Helicobacter pylori* (*H. pylori*) is a risk factor for gastric cancer development. In the multi-step process that leads to gastric cancer, tight junction dysfunction is thought to occur and serve as a risk factor by permitting the permeation of luminal contents across an otherwise tight mucosa. Mechanisms that regulate tight junction function and structure in the normal stomach, or dysfunction in the infected stomach, however, are largely unknown. Although conventional tight junction components are expressed in gastric epithelial cells, claudins regulate paracellular permeability and are likely the target of inflammation or *H. pylori* itself. There are 27 different claudin molecules, each with unique properties that render the mucosa an intact barrier that is permselective in a way that is consistent with cell physiology. Understanding the architecture of tight junctions in the normal stomach and then changes that occur during infection is important but challenging, because most of the reports that catalog claudin expression in gastric cancer pathogenesis are contradictory. Furthermore, the role of *H. pylori* virulence factors, such as cytotoxin-associated gene A and vacoulating cytotoxin, in regulating tight junction dysfunction during infection is inconsistent in different gastric cell lines and *in vivo*, likely because non-gastric epithelial cell cultures were initially used to unravel the details of their effects on the stomach. Hampering further study, as well, is the relative lack of cultured cell models that have tight junction claudins that are consistent with native tissues. This summary will review the current state of knowledge about gastric tight junctions, normally and in *H. pylori* infection, and make predictions about the consequences of claudin reorganization during *H. pylori* infection.

Key words: *Helicobacter pylori*; Tight junction; Claudins; Paracellular permeability; Stomach; Cytotoxin-associated gene A; Vacuolating cytotoxin; Lipopolysaccharide; Urease; Ammonia

Core tip: Tight junction dysfunction is a risk factor for cancer development during *Helicobacter pylori* (*H. pylori*) infection. The recent identification of numerous barrier-forming claudins has greatly improved our understanding of properties that regulate selective permeation across the tight junction in general, but little is known about the role of claudins in the stomach, or in *H. pylori* infection. In this article, we review the current state of knowledge on stomach tight junction composition and organization, discuss the details of claudin expression in various species and in cultured gastric cells, and discuss the implications of tight junction dysregulation in gastric cancer pathogenesis.

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INTRODUCTION

Tight junctions have recently attracted a great deal of interest because of their regulated permeability to ions, solutes, and water conferred by a large and diverse group of transmembrane proteins consisting mainly of occludin, junctional adhesion molecules (JAM's), and members of the claudin family of proteins. Additionally, the discovery that increased intestinal permeability occurs during inflammation by tight junction dysfunction has driven new ways of thinking about the pathogenesis of inflammatory bowel diseases[1-3]. To date, the study of tight junction structure and function in the gastrointestinal tract has focused mainly on intestine and colon, which are considered "leaky" transporting epithelia that take advantage of tight junctions that are selectively permeable to ions and small molecules for passive paracellular absorption and secretion[4,5]. We propose that the stomach is a tighter epithelium that generates favorable ion gradients during active acid and pepsinogen section to drive passive, transcellular transport with little chance of cation (Na^+, H^+) movement across tight junctions. The stomach must act as a barrier to localize toxins, food substances, and the microbiota to the gastric lumen thus inhibiting access to the systemic circulation. The stomach has an additional challenge of limiting secretion-mediated hydrogen ion and pepsinogen back-diffusion across the epithelial barrier. *Helicobacter pylori* (*H. pylori*) infection and its resulting inflammation disrupt the mucosal barrier and thus pose a risk for gastric cancer development[6]. Despite the importance of an intact barrier in the stomach, little is known about the physiology or function of tight junctions in gastric epithelial cells. Our focus in this article is to review current and past work on tight junctions in the stomach and to postulate on their role in disease pathogenesis and cancer development during *H. pylori* infection.

ORGANIZATION OF HUMAN AND MOUSE STOMACH AND GASTRIC GLANDS

The human stomach is organized into four functional regions: (1) the cardia is localized as a ring of cells at the junction of the esophagus and stomach; (2) the fundus and (3) body (corpus) make-up the bulk of the stomach; and (4) the pylorus, consisting of the pyloric antrum and pyloric canal, is the most distal region located proximal to the duodenum (Figure 1). The mouse stomach has body and pylorus regions but additionally has an extensive forestomach consisting of squamous epithelial cells (Figure 1). Gastric glands in both the human and mouse stomach are present in all regions but differ in both cellular composition and in function; cardia and pylorus regions consist mostly of surface and gland mucous cells (not shown), whereas those in the fundus and body consist of surface epithelial cells facing the lumen, gastric pits, which contain mucous-secreting pit cells, and long glands that are further divided into the isthmus, neck, and base containing neck cells, parietal cells, and zymogenic (chief) cells respectively (Figure 2A). Numerous stem cells, committed progenitor cells, and endocrine cells also populate gastric glands in the fundus and body (Figure 2A). Although rarely denoted in schematic diagrams, the specialized epithelial cells in each region possess apical junctional complexes (Figure 2B-D) that consist of occluding and adherens junctions and desmosomes. While adherens junctions and desmosomes primarily function to regulate cell-to-cell adhesion and cell signaling, tight junctions regulate epithelial barrier function and paracellular permeability.

TIGHT JUNCTIONS AND THE MUCOSAL BARRIER IN STOMACH

Tight junctions: General overview

Tight junctions are multi-protein complexes composed of numerous transmembrane and cytoplasmic components that form a continuous structure around the lateral portion of epithelial cells near the luminal surface (Figure 3). By freeze-fracture microscopy analysis of the lateral cell membrane, the tight junction appears as linear rows of straight or anastomosing strands on the P-face (inner surface of the inner lipid monolayer), which likely represent integral membrane proteins of the tight junction, with corresponding grooves on the

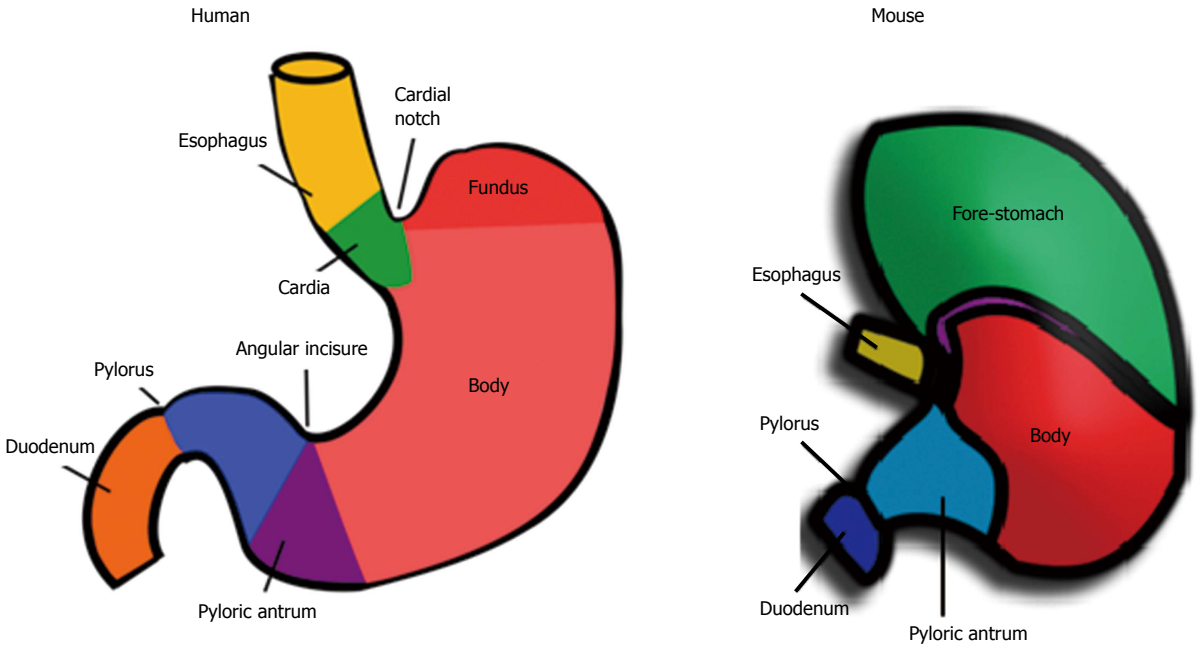

Figure 1 Gross anatomical characteristics of the human and mouse stomach.

E-face (inner surface of the outer lipid monolayer)[7]. The cell-specific strand number and network complexity may be important factors in regulating barrier properties[7]. The outer leaflet of the plasma membrane from adjacent cells additionally have kissing points, which are areas of the membrane that have virtually no intercellular space but rather contain regulated aqueous pores that are thought to function as passive ion channels[5,7,8]. Transmembrane proteins at the tight junction, including occludin and claudins, are associated with tight junction strands. Other transmembrane proteins found at tight junctions include tricellulin, marvelD3 and JAM proteins[9]. Transmembrane proteins are stabilized at tight junctions by peripheral scaffolding proteins such as zonula occludens (ZO)-1, -2, and -3, cingulin, afadin, membrane-associated guanylate kinase with inverted orientation-1 (MAG proteins) and multi-PDZ domain protein 1 (MUPP-1), which are linked to the actin cytoskeleton and to microtubules through numerous linker proteins like non-muscle myosins and cingulin; and a spectrum of associated signaling effectors are found in this macromolecular complex, like Rho, Rac, and cdc42[9]. For a comprehensive description of TJ components, see recent reviews by Van Itallie *et al*[9] and Günzel *et al*[10].

Tight junctions: Regulation of paracellular permeability

Two distinct pathways are involved in the regulation of paracellular permeability at tight junctions. The first pathway is the "pore" pathway, which allows the movement of small molecules, ions, and nutrients through the tight junction along with water. The pore pathway (1) allows charged or uncharged molecules less than approximately 4 angstroms (Å) to cross the tight junction with charge discrimination that is regulated by the expression of claudin molecules; (2) carries most of the electrical current for a given epithelium (reflected in the measurement of transepithelial (electrical) resistance, TER); and (3) regulates the magnitude of permeability and charge selectivity as determinants of tissue-specific physiological transport properties[11]. The second pathway, or "leak" pathway, allows the flux of molecules larger than 4 Å across the tight junction with no charge selectivity that may be due to small temporary breaks in otherwise continuous tight junction strands[11]. This pathway is controlled by cytoskeletal dynamics or factors that affect cell homeostasis[11]. Reports that describe barrier dysfunction in *H. pylori* infection suggest that both pathways are affected; *H. pylori* infection (1) decreases TER and increases permeability, thus affecting the pore pathway including the expression of claudin molecules, claudin composition at tight junctions, and the magnitude of paracellular flux; (2) causes small breaks in tight junction strands and thus increases the flux of sucrose (5.2 Å) and other molecules during infection; and (3) injures gastric epithelial cells, which not only disrupts tight junctions but lateral membrane adherence, in general. The details of each will be discussed below.

Tight junctions: Molecular architecture in gastric epithelial cells

Tight junction structure specific to various parts of the stomach or specific to individual epithelial cell types in the stomach have not been well-characterized. Claude and Goodenough[12] originally classified the mouse stomach as "very tight" because tight junctions had the same number of strands (range, 5-14 strands) described in the urinary bladder, which had a high

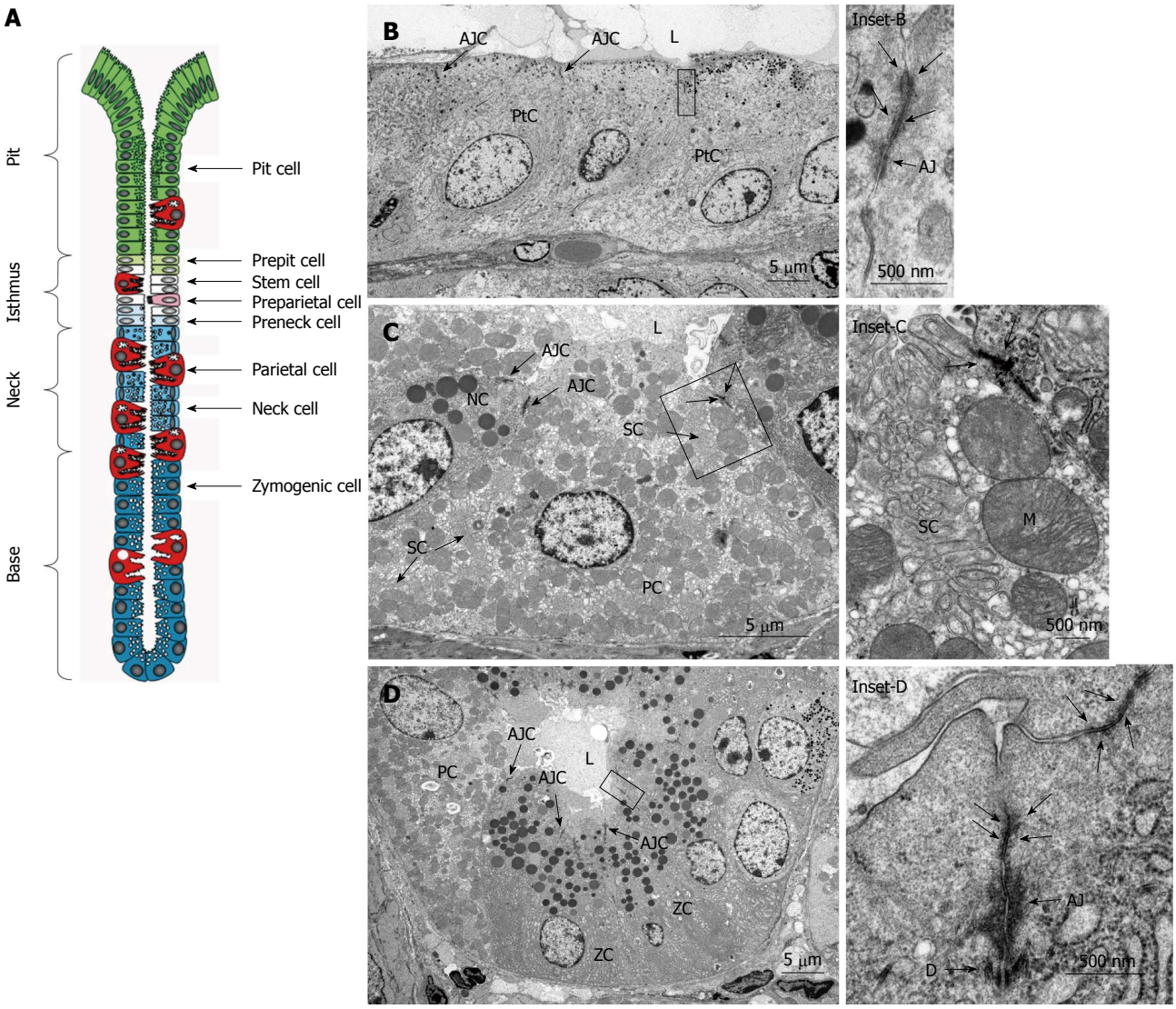

Figure 2 Histological structure of a gastric unit. A: Diagrammatic representation of the organization of a gastric unit (also called a gastric gland), which contains a pit, isthmus, neck, and base. The location-specific cell types are identified in each region. Reproduced with permission from Karam SM[109]; B: Pit region cells (PtC) have apical junctional complexes (AJC) that are near the gastric lumen (L). Inset from the box in B: contains a tight junction (arrows) and an adherens junction (AJ). The desmosome is out of plane in this image. Bar in B is 5 μm and in the inset is 500 nm; C: Neck region cells, consisting mainly of parietal cells (PC) and neck cells (NC) also have AJC near the lumen of the gastric gland (L). Secretory canaliculi (SC) and mitochondria (M) are prominent in parietal cells. Inset from the box in C: contains a tight junction (arrows) and other parts of the apical junctional complex that are out of plane. Bar in C is 5 μm and in the inset is 500 nm; D: Base region cells consist mostly of zymogenic cells (ZCs) and a few PCs that also have AJC. Note that similar to the diagram in A, the apical cell cytoplasm of the zymogenic cells extends as a triangular wedge into the gland lumen (L) and apical junctional complexes are found at the lateral membranes where cells meet. Inset from the box in D: contains an apical junctional complex consisting of the tight junction (arrows), AJ, and desmome (D). Bar in D is 5 μm and in the inset is 500 nm.

TER of 1000-2000 Ohm·cm^2. Structural differences were then described in tight junction strands in various parts of the gastric unit[13], suggesting that the tightness and transport properties of tight junctions are different at the surface and in gastric glands. As an example, tight junctions in surface epithelial cells are composed of 5 to 6 strands that are woven together into a deep, honeycomb-type structure whereas the same number of strands in cells from gastric glands (both parietal and chief cells) are organized in a shallow, regular, linear configuration[13]. Of particular note, however, was the difference in permeability in the two regions. When lanthanum (La^{3+}) was instilled into the gastric lumen during fixation, this small (4.2 Å) electron-dense molecule was unable to cross tight junctions and was thus excluded from the basolateral intercellular space between adjoining surface epithelial cells[13]. In contrast, La^{3+} was frequently found within the basolateral membrane space in gastric glands, particularly surrounding parietal cells[13]. Another novel finding specific to stomach were structures resembling tight junctions along the basolateral membrane of epithelial cells by freeze fracture microscopy[12]; the structures described were discontinuous and were proposed to be unrelated to the regulation of epithelial permeability[12]. Overall, these interesting findings suggests that epithelial cells in gastric glands, compared to surface epithelial cells, are particularly permeable to

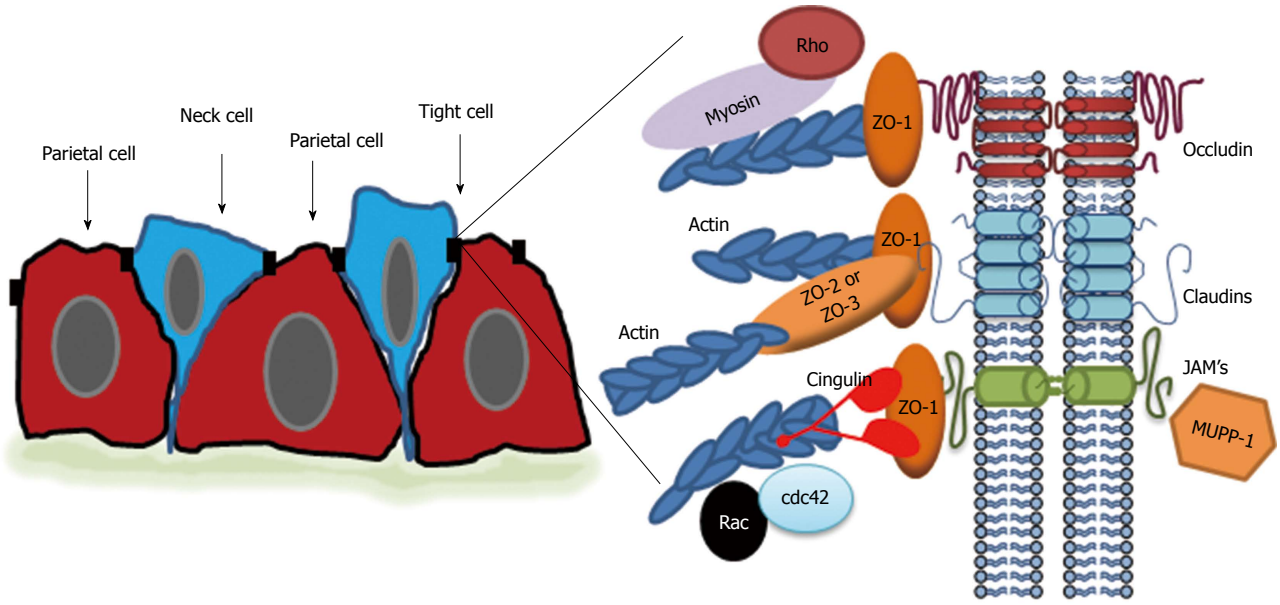

Figure 3 Schematic representation of the tight junction in gastric epithelial cells. In the neck region of gastric glands, neck and parietal cells are oddly shaped but make tight junctions at the apical border between cells. Expanded diagram: Tight junctions in the stomach have classical components consisting of transmembrane proteins including occludin, claudins, and JAM proteins; peripheral scaffolding proteins like zonula occludens (ZO)-1, 2, and 3; linker proteins to the actin cytoskeleton like non-muscle myosin and cingulin; and signaling molecules like Rho, Rac, and cdc42. Actin filaments are also prominent.

small ions due to the composition and organization of tight junction strands, and that novel, tight junction-like structures may also be a feature of the basolateral membrane of gastric epithelial cells.

Gastric epithelial cells contain transmembrane proteins, like occludin, JAM-A, and claudins[14-19], and peripheral scaffolding proteins, like ZO-1[17] (Figure 3). In the human stomach, immunostaining for occludin appeared to localize at the tight junction and along the basolateral membrane[16], but because there was no accompanying control to evaluate non-specific staining it is not clear if this result truly reflects the localization of occludin *in vivo*. Tricellulin has been localized to tricellular contacts within tight junctions in epithelial cells of human stomach[16,20]. There was also considerable lateral membrane staining for tricellulin; however, without controls for non-specific background it is not clear whether the lateral membrane staining is specific.

Little is known about the differential expression of claudin proteins in tight junctions of normal gastric epithelial cells. Because claudins localize to tight junction strands and because the strand configuration is different in surface cells compared to the cells in gastric glands, it may be important to determine the claudin footprint of each cell-type or area to better understand the details of permselectivity in the stomach.

Claudins and selective permeability or "permselectivity"

Although the "tightness" or "leakiness" of tight junctions was originally proposed to be determined by the number and depth of tight junction strands[7,12], knowledge about the presence of tight junction claudins currently dominates our thinking about the regulation of tight junction permeability and permselectivity. Although occludin is expressed in stomach epithelial cells and is membrane-spanning at tight junctions, this protein is not involved in the regulation of paracellular permeability in the stomach[15]. In contrast, claudin proteins are expressed in the stomach and are likely to determine epithelial permeability and permselectivity.

In mammals, the claudin family of proteins currently consists of 27 different tetraspanning proteins that are normally expressed in a tissue dependent fashion[10,21]. Claudins associate with a host of other cytoplasmic and extracellular proteins, and play roles in the regulation of tight junction permeability, cell signaling, cell cycle regulation, the maintenance of cell polarity, and vesicle trafficking[10]. The crystal structure of mammalian claudin 15 was recently determined, revealing four underlying transmembrane helices which anchor a unique extracellular beta-sheet fold made from the first and second extracellular loops[8,22]. These extracellular loops contain 5 beta strands which, when aligned properly into 2 continuous antiparallel rows, have been proposed to form "half-pore" structures, each containing two variable regions which, when aligned with adjacent cells, form complete TJ pores[8]. The model, which was described by Suzuki *et al*[8], suggests that the charges possessed by two intra-pore variable regions in each claudin dimer determine the selective permeability characteristics. Just as the specific claudin populations expressed in different epithelial cell types are thought to determine tissue specific solute permeability, altered claudin expression

has been linked to a number of pathologic conditions including gastric cancer (discussed below).

Claudin expression in the stomach-an overview

Human stomach: By genetic analysis using the serial analysis of gene expression (SAGE) database followed by RT-PCR techniques, the expression of claudins 1-5, 7-12, 16 and 18 have been demonstrated in normal human stomach (Table 1). Bioinformatics approaches combined with genome-wide analyses also identified claudins 21-23 in the human stomach (Table 1).

Immunostaining analysis in human stomach demonstrated the expression of claudin 1, 3-5, 7, 10, 14, and 18 (Table 1). Claudin 1 expression was high in epithelial cells from both corpus and antrum, whereas the expression of claudins 3, 4, and 5 was stronger in corpus compared to antrum (Table 1). In other work, immunostaining analysis of human tissues concluded that weak to no claudin 3 or 4 expression was present in the normal gastric mucosa (Table 1). Normal stomach tissues adjacent to gastric tumors demonstrated claudin 1 expression in about 50% of tissues, claudin 3 expression in about 24% of tissues, and claudin 4 expression in about 15%[23,24] to as high as 40%-50% of tissues[17,25,26]. Similarly, claudins 2, 6, and 11 were expressed in 68%, 79% and 46% of tissues, respectively[27]. These results suggest that the differing results in studies from human patient gastric samples might be explained, at least in part, by the source and/or location of "normal" tissues used in for immunostaining. As for location and cell specificity in human studies, claudin 1 was found to be strongly expressed in gastric surface epithelial cells and chief cells whereas it was weakly expressed in parietal cells (Table 1). Other than the localization of claudin 18 (below), the localization of other claudins to specific epithelial cell types in the corpus or antrum is unknown (Table 1).

Canine stomach: A recent immunohistochemical analysis of claudin expression in the normal canine stomach revealed a robust basolateral membrane localization of claudin 18 in all fundic epithelial cells[28]. In the pylorus, all glandular cells expressed claudin 18, while only basally located glandular cells expressed claudin 2[28]. Surface, mucous neck, parietal, chief, and endocrine cells of the fundus, as well as surface and glandular cells in the pylorus were negative for claudins 1, 3-8 and 10[28].

Rat stomach: Immunohistochemical analysis of claudins 2, 3, 4, and 5 expression in Sprague Dawley rats showed that there was no difference in the expression level or cellular localization of these specific claudin in any region of the stomach; claudin 3 was most highly expressed at the basolateral membrane of surface epithelial cells, without enrichment at the tight junction[29]. Similarly, claudin 5 was localized to the basolateral membrane of all cells comprising the gastric glands[29]. The only tight junction protein identified to localize at the tight junction *per se*, with no basolateral expression, was claudin 4, which also showed higher expression in proximal gastric glands[29]. Claudin 2 expression was not detected in any part of the stomach[29].

Mouse stomach: By quantitative RT-PCR, claudins 1, 3, 4, 6, 7, 10, 12, 15, 17, 18, 23, and 25 are expressed in the stomach of neonatal C57BL/6 mice[14]. By immunostaining, claudins 1, 3, 5, and 18 are expressed in the glandular stomach while claudins 6 and 11 are confined to the squamous fore-stomach[30]. Claudins 1 and 3 are localized to the basolateral membrane of epithelial cells in the glandular stomach whereas claudins 5 and 18 are basolateral but appear to be enriched at tight junctions[30]. In the stomach from adult C57BL/6 mice, low levels of all claudin -family members were present, as determined by quantitative RT-PCR analysis[14,31], but claudin 18 was expressed at a level considerably higher than all other claudin-family members[14]. Immunostaining studies in adult mouse stomach consistently showed that claudin 2 expression was negative in corpus epithelial cells but present at the base of antral glands[32], similar to the canine stomach[28].

Claudin 18 expression in gastric epithelial cells

Claudin 18 is likely to be the most important barrier-forming claudin family member in the stomach because its expression is 30-fold or more greater than all other claudins, at least in mouse stomach[14]. In general, claudin 18 has four differentially expressed isoforms; claudin 18A1.1 and A1.2 are expressed almost exclusively in lung and claudin 18A2.1 and 18A2.2 are expressed almost exclusively in the stomach, with claudin 18A1.1 highly expressed and claudin 18A.2 barely present[33]. Additionally, claudin 18A2.1 localizes to the basolateral membrane of gastric epithelial cells rather than being concentrated solely at tight junctions[14,33,34]. When transfected into Madin-Darby canine kidney epithelial (MDCK) cells, claudin-18 raised electrical resistance and significantly reduced the paracellular permeability to cations, specifically Na^+ and H^+[35], suggesting that claudin-18 functions as a strong cation exclusion pore at tight junctions. Knockout mice (C57BL/6) deficient in *C18A2.1* confirmed the importance of this claudin in stomach, given the knockout mice displayed an increase in paracellular H^+ leakage as well as transepithelial conductance. Additionally, inflammation was present and the mice rapidly developed atrophic gastritis due, in part, to H^+ back-diffusion and mucosal injury[14]. In human studies (Table 1), the attenuation of claudin 18 expression in the gastric mucosa was prominent in early GC development, and predicted an unfavorable outcome after cancer diagnosis[34,36-38]. Although these studies suggest that a loss of gastric epithelial claudin 18 leads to the stepwise development

Table 1 Claudin expression in the normal human stomach and changes that occur in gastric cancer

	Location	Detection method	Expression (normal stomach)	Patient outcome	Changes in GC
Cldn 1	Unspecified region	SAGE database and RT-PCR[110]	Present	Not evaluated	Present, no change in GC
	Unspecified region	cDNA oligonucleotide microarray analysis[67]	Present	Up-regulation results in extremely poor outcome	One of the most highly up-regulated genes
	Corpus, antrum	Immunostaining[69]	Strong expression in epithelial cells	No association with patient outcome	Some GC with strong expression and some with no expression
	Antrum	Immunostaining[74]	Strong expression in epithelial cells	No association with patient outcome	No change in expression in GC
	Unspecified region	Immunostaining[68,70]	Not evaluated	Not evaluated	Highly expressed in GC; most highly expressed at invasive front
	Unspecified region	Immunostaining[25]	Tumor margin	Not evaluated	55.4% of cells are positive at the tumor margin Reduced expression in GC
	Corpus	Immunostaining[73]	Surface and chief cells ++++; parietal cells +	Not evaluated	Basolateral localization Expression in GC is dependent on the expression of RUNX3
Cldn 2	Unspecified region	qRT-PCR[111]	Weak expression	Not evaluated	No change in GC
	Unspecified region	cDNA oligonucleotide microarray analysis[67]	Present	Not evaluated	Highly up-regulated in GC
	Unspecified region	Immunostaining[68,83]	Not evaluated	Not evaluated	Highly expressed in GC
Cldn 3	Unspecified region	SAGE database and RT-PCR[110]	Present	Not evaluated	Up-regulated in GC
	Unspecified region	Immunostaining[112]	Not evaluated	Not evaluated	Higher expression in low grade compared to high-grade malignancy
	Unspecified region	Immunostaining[25,36,68,113-116]	Low to no expression in stomach	Up-regulation has no effect on survival	Highly expressed in the majority of GC's
				Up-regulation associated with a significantly higher incidence of synchronous and metachronous multiple GC and gastric adenomas[114]	Increase in expression occurs in metaplasia
	Corpus, antrum	Immunostaining[69]	Corpus, strong expression; Antrum, weaker expression	Strong expression results in better outcome.	Some GC with strong expression and some with no expression
	Antrum	Immunostaining[74]	No expression	No association with patient outcome	Most GC weak to moderate expression
Cldn 4	Unspecified region	SAGE database and RT-PCR[17,110]	Present	Not evaluated	Highly up-regulated in GC
	Unspecified region	Immunostaining[112]	Not evaluated	Not evaluated	Higher expression in low grade compared to high-grade malignancy
	Unspecified region	Immunostaining[17,23-26,68,113-117]	Low to no expression in stomach	No association with patient outcome.	Highly expressed in GC Localized to the basolateral membrane Prominent in intestinal-type GC
	Unspecified region	Immunostaining[126]	Low to no expression in stomach	High expression is associated with favorable prognosis and longer survival; low expression is associated with poor survival	Highly expressed from stages intestinal metaplasia to GC Localized to the basolateral membrane
	Corpus, antrum	Immunostaining[69]	Corpus, strong expression; Antrum, weak expression	No association with patient outcome	Strong expression associated with metaplasia Some GC with strong expression and some with no expression
	Antrum	Immunostaining[74]	No expression	High expression associated with poor outcome	Intestinal metaplasia highly expressed 90% of GC have weak to strong expression

Cldn 5	Unspecified region	SAGE database and RT-PCR[110]	Present	Not evaluated	Present, no change in GC
	Corpus, antrum	Immunostaining[69]	Corpus, strong expression; Antrum weak expression.	No association with patient outcome.	Some GC with strong expression and some with weak expression.
Cldn 7	Unspecified region	SAGE database and RT-PCR[110]	Present	Not evaluated	Highly up-regulated in GC
	Unspecified region	cDNA oligonucleotide microarray analysis[67]	Present	Not evaluated	Highly up-regulated in GC
	Unspecified region	Immunostaining[36]	Not present in stomach	Up-regulation correlated with poor survival	Highly up-regulated in GC
Cldns 8-12	Unspecified region	SAGE database and RT-PCR[110]	Present	Not evaluated	Present, no change in GC
Cldn 10	Unspecified region	Immunostaining[118]	Highly expressed	Not evaluated	Significantly reduced in GC
Cldn 11	Unspecified region	RT-PCR methylation analysis[119]	Not evaluated	Not evaluated	Highly methylated in gastric cancer, which is correlated to attenuated expression.
Cldn 14	Unspecified region	Immunostaining[118]	Little to no expression	Not evaluated	Highly expressed in GC Localization to the basolateral membrane
Cldn 16	Unspecified region	SAGE database and RT-PCR	No expression	Not evaluated	No expression in GC
Cldn 17	Unspecified region	Immunostaining[118]	Highly expressed	Not evaluated	Significantly reduced in GC
Cldn 18	Unspecified region	SAGE database and/or RT-PCR[110,34,36]	Not evaluated	Down-regulation correlated with poor survival	Identified as a highly expressed gene that is significantly down-regulated in GC
	Unspecified region	RT-PCR[34,120]	Not evaluated	Not evaluated	Cldn18A1 is not expressed in stomach or in GC whereas Cldn18A2 is expressed in stomach and in some GC's
	Corpus or antrum	Immunostaining[34,37,113]	Surface, ++++ Pit, + Parietal/Neck, +++ Zymogenic, +++	Down-regulation correlated with poor survival	Basolateral localization. Attenuation is an early event, which occurs in the metaplastic mucosa
Cldns 21, 22, 23, 24	Database search	Bioinformatics[121]	Not evaluated	Not evaluated	Identified genes for Cldns 21-24
Cldn 23	Unspecified region	Genome-wide analysis[122]	Not evaluated	Not evaluated	Cldn-23 down-regulated in 78.9% of GC with an intestinal phenotype

++++, very highly expressed; +, weak expression. Cldn: Claudin; GC: Gastric cancer.

of chronic inflammation and gastric cancer, it has not been shown that the expression of claudin 18 is affected by *H. pylori* infection nor has it been shown that the attenuation of claudin 18 results in cancer development. Thus, direct cause-and-effect evidence is lacking. Additionally, it is possible that other claudins with similar cation-exclusion function might be upregulated to compensate for the lack of claudin 18 expression in GC development.

HELICOBACTER PYLORI: DISEASE PARAMETERS THAT AFFECT BARRIER DYSFUNCTION - OVERVIEW

H. pylori is a highly adapted, spiral shaped, gram negative bacteria that colonizes the human stomach, with animal-adapted cultivars that infect the non-human primate, cat, mouse, guinea pig, gerbil, and rat stomach[39]. The bacterium is catalase and oxidase positive, microaerophilic, and possesses 3 to 5 polar sheathed flagella that are used for motility[40]. *H. pylori* also possess urea transporters that are utilized during acid exposure, in conjunction with urease, to neutralize pH, and support colonization[41,42]. Both *H. pylori* and ammonia have been identified as important factors that regulate barrier dysfunction in *H. pylori* infection, the details of which can be found in the section on cultured cells, below. *H. pylori* are also associated with a specific set of virulence factors, including VacA and the cytotoxin-associated gene pathogenicity island (Cag PAI), which codes for a type 4 secretion system that delivers CagA into the cytoplasm of epithelial cells. Both VacA and CagA are considered important *H. pylori* virulence factors that regulate disease pathogenesis and barrier dysfunction. The role of VacA and CagA on *H. pylori*-induced barrier dysfunction is also discussed in detail below in the section on cultured cells. Lipopolysaccharide (LPS) from *H. pylori* is also considered toxic to gastric epithelial cells[43]. While *H. pylori* LPS is considered less potent than the LPS from other bacteria, like *E. coli*[43], it decreases the TER and increases the permeability of cultured primary gastric

epithelial cells[44]. Interestingly, this effect is greater from the basolateral compared to apical surface[44] suggesting that LPS may be most effective at damaging the mucosal barrier if it is able to permeate the mucosa and gain access to the basolateral surface of gastric cells.

H. pylori is categorized as a type 1 carcinogen by the World Health Organization and International Agency for Research on Cancer[45], and is responsible for a large percentage of gastric cancer, which is the fifth most common cancer and the third most common cause of cancer-related deaths worldwide[46,47]. Without therapeutic intervention, *H. pylori* infection leads to a persistent, life-long infection. *H. pylori* infection of the gastric corpus is associated with intestinal-type gastric adenocarcinoma and initiates a well-defined pathological process, referred to as the "Correa Cascade", which is characterized by chronic superficial gastritis followed by atrophic gastritis and intestinal metaplasia, which progresses to dysplasia and adenocarcinoma[48,49]. Although eradication of *H. pylori* appears to be the most feasible approach to reducing GC rates, previous studies were recently reviewed by Lu *et al*[50] and suggest otherwise. Furthermore, eradication of *H. pylori* after endoscopic resection of tumors does not reduce the development of metachronous gastric carcinoma[51], suggesting that either *H. pylori*- or inflammation-induced genetic and epigenetic changes in gastric epithelial cells, microsattelite instability, or other permanent changes occur in the stomach that cannot be reversed by bacterial eradication. Changes in the expression of tight junction components may be part of this global pattern of gene expression changes that impact cancer pathogenesis.

DISRUPTION OF TIGHT JUNCTIONS IN *H. PYLORI* INFECTION

Early studies using electron microscopy and human biopsy samples from patients infected with *Campylobactyer pylori*, the organism later renamed *H. pylori*, demonstrated that *C. pylori* colonize the gastric mucosa and are highly concentrated along the luminal surface of surface mucous cells proximal to tight junctions[52]. They also migrate between epithelial cells[53]. The bacteria target surface epithelial cells that express *Limax flavus* agglutinin, which is a lectin that is specific for sialic acid-rich glycoproteins, and adhere to the surface by making intimate contacts that result in the depletion of microvilli and alterations in the cell including dissolution of apical secretory granules and rounding of the apical cell surface[52]. *C. pylori* were also found to penetrate the apical junctional complex[53]. Later Necchi *et al*[54], using tissues from *H. pylori* infected patients, confirmed these initial findings and extended them to show that junctional penetration occurs in both antrum and corpus, and demonstrated that although most of the tight junctions were intact, there were some cells with detached junctional complexes that contained $CagA^+VacA^+$ *H. pylori* traversing the intercellular space and residing near the base of cells. Noach *et al*[55] described that the majority of *H. pylori* were scattered in the mucous layer or positioned at the aforementioned tight junction location without cell contact but some bacteria formed adhesion pedestals at the bacterial and cell interface and were seen entering cells, which occurred next to tight junctions. Further work, reviewed by Fox *et al*[56], described that most of the bacteria in the infected stomach exist in a non-adherent configuration in the extracellular mucous environment. By freeze fracture microscopy, uninfected antral epithelial cells had a thin tight junction area consisting of about four strands that formed interconnecting ridges whereas the tight junction region from *H. pylori*-infected patients was significantly deeper with irregular, knobby, and locally fragmented strands[55]. Tannic acid, which was used to evaluate live and dead cells in tissues from *H. pylori*-infected patients, demonstrated that many of the cells from infected *vs* control patients were tannic acid-positive, but additionally, the number of tannic acid-containing cells was associated with the inflammatory score of the specimen[57]. Sucrose permeability and the permeability of food antigens increased in patients with *H. pylori* infection[58-60], supporting the notion that barrier dysfunction accompanies defects in tight junction structure during infection. Animal models also support this idea; C57BL/6 mice infected with *H. pylori* Sidney strain 1 (SS1) showed a 30-fold increase in lanthanium (4.2 Å) permeability across tight junctions into the intercellular space *in vivo*[61], and an increase in sucrose (4.6 Å) excretion, *in vivo*, was also found in infected mice[62]. In the later study, permeability defects occurred only transiently, at about 12 wk post-infection, whereas permeability before 12 wk and up to 100 d post-infection was not significantly different from control mice[62]. The reason for this result is unknown. In *H. felis*-infected C57BL/6 mice, Ussing chamber experiments were used to demonstrate that infected mice also increased antral HRP flux (30 Å)[63]. Overall, these results suggest that both tight junction pore and leak pathways are affected during *H. pylori* infection, resulting in an increase in small molecule permeation across tight junctions and an increase in the permeation of larger molecules that may be due to small temporary breaks in tight junction strands. Alternatively the leak pathway alone may be activated by defects in cytoskeletal dynamics, particularly MLCK activity, consistent with the results obtained in cultured cells by Wroblewski *et al*[64]. Interestingly, HRP flux across tight junctions by electron microscopy correlated with transitional zone neutrophilic gastritis[61] and eradication of *H. felis* infection in C57BL/6 mice returned the HRP permeability defect to normal if inflammation concomitantly decreased[63]. These results suggest that inflammation may be the most important

component of tight junction and permeability defects in *H. pylori* infection.

Inflammation and gastric tight junction dysfunction

Few studies have been done to address the role of inflammatory cytokines on tight junction dysfunction in the stomach or in model gastric epithelial cells. Gastric HGE-20 cells were initially used to show that IL-1 receptor phosphorylation by interleukin (IL)-1β occurs after exposure to *H. pylori*, resulting in the reduction of claudin 4 expression that seems to be internalized into the cell cytoplasm[65]. This work was not accompanied, however, by TER or permeability studies, so it is unclear whether or not exposure to IL-1β results in barrier dysfunction or just targets specific claudins for degredation. It is also unclear how IL-1 receptor phosphorylation occurred after exposure to *H. pylori* in cultured cells because immune cells were not present in the assay; whether some aspect of infection-mediated signaling activated the receptor pathway or if the cells secreted IL-1β, which self-activated the receptor. To address this issue using NCI-N87 cells, Fiorentino *et al*[66] showed that exposure to *H. pylori* reduced TER and increased paracellular permeability over time without a reduction in cell viability but with a concomitant increase in cytokine production by epithelial cells, including IL-8, IL-6, interferon (IFN)-γ, IL-1β, tumor necrosis factor (TNF)-α, and IL-10, which increased with either live or heat-killed bacteria. On the other hand, barrier dysfunction was accompanied by the reorganization of ZO-1 and claudin 1 proteins, but required live bacteria[66]. Thus, a cause and effect relationship between cytokine production and barrier defects in NCI-N87 cells was not fully established nor was the role of any particular cytokine further investigated to determine which would be involved in barrier dysfunction. These preliminary studies, in addition to the seminal studies on permeability in human and animal models, provide justification for further studies examining the role of pro-inflammatory cytokines in modulating tight junction dysfunction during infection. It would be very interesting to determine, in particular, if inflammation modulates the expression of claudin molecules that are normally responsible for maintaining the gastric mucosal barrier.

Claudin protein expression in H. pylori infection-mediated GC

There has been considerable work done to catalog the changes in claudin expression in human gastric cancer caused by *H. pylori* infection (Table 1). These results, however, are difficult to interpret because the results from different groups, who are evaluating the expression of the same claudin, may be completely opposite, making it difficult to elucidate the role of claudins in cancer pathogenesis. As an example, Claudin 1 expression (Table 1) was found to be significantly lower in GC tissue *vs* adjacent tissues with no correlation to histological grade in one study[25], but in another study it was highly expressed in GC, as one of the most differentially upregulated genes in gastric tumors *vs* control tissues[67]. Similarly, in one study (Table 1) claudin 1 expression was more prominent overall in diffuse compared to intestinal GC[68] but in another study showed less expression in diffuse *vs* intestinal-type GC[69]. Claudin 1 expression was reported to be highest at the invasive front of GC, being highest in well-to-moderately differentiated carcinomas and lowest in poorly-differentiated carcinomas[70]. These studies were consistent with some cell culture experiments (Table 1), which demonstrated that over-expression of claudin 1 increased the migration and invasion of cultured gastric cells[71,72], but in contrast to others (Table 1), which demonstrated that the attenuation of claudin 1 increased migration and invasion[73]. The later study went-on to conclude that normally high expression levels of claudin 1 function as a tumor suppressor[73]. In some studies (Table 1), the expression of claudin 1 was not associated with patient outcome[69,74], whereas in another report it was highly correlated to patient outcome, with cumulative survival rates of 0% at about 12 postoperative months for patients with high claudin 1 expression *vs* about 50% survival at 50 mo for patients with low claudin 1 expression[67]. Each study included a significant number of patients, had control staining to verify the antibody, but found significantly different results. For patient studies to be meaningful, it appears that there must be guidelines adopted for study design to avoid conflicting results. What seems to be consistent between studies is that claudins 2, 3, 4, and 7 are highly upregulated, whereas claudin 18 is down-regulated in gastric cancer (Table 1). Recent studies have also evaluated less well-known claudins, such as claudins 10, 11, 14, 17, and 23 and found that claudins 10, 11, 17, and 23 were down-regulated and claudin 14 was highly up-regulated in patients with gastric cancer (Table 1). For a comprehensive review of claudin expression in gastric carcinogenesis, see Iravani *et al*[75].

Claudin expression changes in GC, including the attenuation of claudins 11 and 18, which normally produce a tight barrier[11], and an increase in claudin 2, which would significantly increases the paracellular cation leak[11], suggest that the paracellular barrier would be leaky in GC tissues, particularly to cations like Na^+ and H^+. This may be particularly detrimental in the stomach, which normally functions to limit H^+ back-diffusion from the lumen but furthermore, must maintain an effective luminal to basolateral Na^+ gradient so that ion transport functions can occur for pH regulation and for H^+ and bicarbonate secretion. It is possible that the upregulation of claudins 3, 4, 7, and 14, which function to tighten the barrier[11], and in particular claudin 14, which forms a strong cation exclusion channel[5], may increase as a means to compensate for the attenuation of claudin 18; in an attempt to regain barrier function and permselectivity in the absence of claudin 18. Claudins are also

known to have other functions in addition to acting at tight junctions to regulate permeability. Claudin 7, which is highly upregulated in GC progression in both humans (Table 1) and in *Helicobacter felis*-infected insulin-gastrin (INS-GAS) transgenic mice (FVB/N background)[76], may function in a protective manner to regulate ion transport and regain NaCl homeostasis as described for intestine[77], or it may drive tumorgenesis by binding to epithelial cell adhesion molecule (EpCAM) and regulating cancer pathogenesis[78,79]. Further studies would be required to resolve these two interesting possibilities.

STUDYING *H. PYLORI*-INDUCED TIGHT JUNCTION DYSFUNCTION IN CULTURED GASTRIC AND OTHER CELLS

One challenge in studying gastric barrier function in reductionist models is the lack of gastric-specific cell lines that form a confluent monolayer with a robust luminal to serosal permeability barrier. Most of the cell lines available for studying the gastric mucosal barrier lack or do not have a completely profiled inventory of TJ components, do not grow in monolayers, and do not express claudin 18 (Table 2). The human NCI-N87 gastric cancer cell line is one exception, in that it forms a confluent cell monolayer, expresses claudin 18 (Table 2), and has a transepithelial resistance (TER) of about 1000 Ohm cm^2[66] compared to MKN28 cells, which can be induced to form a confluent monolayer but without a significant TER[64]. Clones isolated from NCI-N87 cells, in particular the HGE-20 clone (Table 2), grow in a confluent monolayer that is polarized, have apical junctional complexes that express ZO-1, express some markers of prezymogenic cells, and have a TER of about 200 Ohm cm^2[80,81]. Gastric adenocarcinoma AGS cells form a confluent monolayer that express ZO-1 and numerous claudins (Table 2), but lack the ability to form functional TJs when grown in monolayers. Because of these important challenges, AGS cells are often used in conjunction with other cells lines for studies concerning gastric barrier function[62,66,82-84]. Limited by the lack of appropriate gastric cell lines, most studies have used intestinal, colonic, or kidney cell lines, including SCBN cells[66], MDCK cells[84,85], T-84[86], or Caco-2[58,87] cells to unravel mechanisms related to the role of *H. pylori* in barrier dysfunction. Gastric organoids from human biopsy samples[88] and primary human cultured cells from biopsy samples[89,90] are also viable options; they each form a monolayer of native gastric epithelial cells and have been shown to express occludin or ZO-1 at cell junctions. However, the compliment of other tight junction components including claudins has not been determined.

CagA: Disruption of the tight junction complex with CagA was first studied in MDCK cells, which clearly demonstrated TJ disruption including relocation of ZO-1 and JAM-A to bacterial adherence sites[82], and the mislocalization of ZO-1 to the basolateral membrane[91]. AGS cells that were incubated with Cag^+ *H. pylori* had severely damaged tight junctions and the presence of CagA resulted in cell scattering and a migratory phenotype consistent with the results in MDCK cells[89,92]. The same occurred in primary human cells that were cultured from antral mucosa[89]. In AGS cells, treatment with $CagA^+$ *H. pylori* also upregulated caudal type homeobox 2 (CDX2) and claudin 2 expression so it was concluded that CagA disrupts tight junctions by targeting claudin 2[92]. This is an interesting premise, however, because claudin 2 forms aqueous pores that are permeable to small cations[5] but does not cause tight junctions to form wide gaps and otherwise disassociate. CagA from *H. pylori* also localized with ZO-1 at tight junctions in T84 cells and over time, resulted in the enrichment of claudin 4[93], suggesting that the transcriptional regulation of both claudin 2 and claudin 4 in *H. pyori* infection is *via* CagA. Work done jointly with MDCK and AGS cells were used to demonstrate that CagA specifically targets polarity-regulating kinase partitioning-defective 1b (Par1b)/MAP/microtubule affinity-regulated kinase 2 (MARK2) to disrupt apical tight junctions[94,95], reduce TER[95], and cause ZO-1 to disassemble from junctions[95]. It is interesting, however, that $CagA^+$ *H. pylori* did not affect tight junctions in Caco-2 intestinal cells[87] or in HGE-20 or MKN28 cultured human gastric epithelial cells[64,65], in the human stomach, *in vivo*[54,65] or in mouse models of *H. pylori* infection *in vivo*[96,97]. To address the apparent differences *in vivo*, interesting experiments done recently in *Drosophila* identified numerous genetic modifiers of Cag-A induced epithelial disruption[98]. From a total of 10 genes whose expression significantly attenuates the effects of CagA were Lasp and chitinase 1[98], both of which are highly expressed in gastric epithelial cells. Lasp-1 is highly expressed in parietal cells as a component of the actin cytoskeleton[99,100] and its activity is regulated by gastrin[101], which is an important effector in *H. pylori* infection[102]. Chitinase 1 is also expressed in human and mouse stomach[103] but has a relatively unknown function. Overall these results suggest that CagA specifically targets components of the tight junction in addition to regulating the transcriptional program of gastric cells *in vitro*, but that the intact mucosa may express important modifiers that regulate CagA function to limit damage and preserve barrier function *in vivo*.

VacA: Disruption of the tight junction barrier by purified VacA from *H. pylori* was also done initially in MDCK cells[84], demonstrating that TER declined in a pH-dependent manner when acid-activated toxin was used but that the decrement in TER did not occur by disrupting the integrity of tight junctions[84]. It was additionally shown that acid activation of VacA resulted in a pronounced increase in the permeability of mannitol and sucrose but not of inulin or HRP[84].

Table 2 Characteristics of human gastric cell lines that are used to study the role of Helicobacter pylori in tight junction dysfunction during infection

Cultured cell line	ZO-1, 2, or 3	Occludin	JAM's	Tricellulin	Cldns	Confluency	Ref.	Cldns not expressed
AGS	ZO-1				2, 4, 6, 7, 9	Confluent monolayer with no TER	[82,92,123-126]	11[119]
BGC-823			JAM-A (low)		1, 18		[19,38,71]	
GES-1			JAM-A (high)				[19]	
HFE-145					11		[119]	
HGE-20	ZO-1				4	Confluent monolayer with TER-polarized	[65,80,81]	
HS-746T					1		[71]	
HSC-39					2		[111]	18[34]
HSC-45				Yes			[16]	
HSC-57				Yes			[16]	
HSC-59				Yes			[16]	
KATOIII	ZO-1	Yes			1, 2, 4, 18		[38,71,72,111, 126,127]	11[119], 18[34]
MKN-1								4[126], 18[34]
MKN-7	ZO-1	Yes		Yes	4		[16,127]	18[34]
MKN-28	ZO-1	Yes	JAM-A		1, 3, 4, 7	Confluent monolayer with a TER	[64,71,72,126-128]	11[119], 18[34]
MKN-45	ZO-1	Yes			1, 2, 3, 4, 18	Isolated cell clumps	[34,71,72,108,111,127]	
MKN-74				Yes	2, 4, 18		[16,34,37,38,111,126]	18[108]
MUGC4					1		[72]	
NCI-N87	ZO-1				1, 4, 18	Confluent monolayer with a moderate TER	[38,66,71,126]	
NUGC-3								18[108]
SIIA								11[119]
SCG-7901			JAM-A		1, 18		[19,38,71]	
SNU-1								11[119]
SNU-5					4		[126]	
SNU-216					4		[126]	
SNU-484								4[126]
SNU-601					4		[126]	
SNU-620					4		[126]	
SNU-638								4[126]
SNU-668								4[126]
SNU-719					4		[126]	
TMK-1	ZO-1				1, 3, 4, 7, 12, 15, 18		[129]	18[34]

Cldn: Claudin; ZO: Zonula occludens; TER: Transepithelial (electrical) resistance.

Moreover, VacA increased permeability to anions[84]. Pelicic *et al*[85] extended these findings to include *H. pylori*, and using the VacA$^+$CagA$^+$ strain CCUG17874 and an isogenic VacA mutant demonstrated that VacA accounted for the entire decline in TER and increase in mannitol flux in MDCK cells. Overall these results suggested that VacA affects the tight junction pore pathway by increasing pore size and thus paracellular transport of small molecules (mannitol, 3.6 Å and sucrose 4.6 Å), but not the leak pathway, which would enhance the permeability of large molecules like inulin (11.5 Å) and HRP (24 Å)[11]. These results also suggested that changes in claudin expression occurred to increase the magnitude of flux through tight junctions in addition to changing permselectivity. In contrast to studies using MDCK cells, compelling results were obtained using the gastric NCI-N87 cell line; the TER was reduced with *H. pylori* and with each of the cytotoxin-associated isogenic mutants including VacA, CagA and urease subunit B (ureB), suggesting that barrier dysfunction occurs in *H. pylori* infection independent of the associated virulence factors including VacA[66]. Caco-2 cells[87] and MKN28 cells[64] are additionally unaffected by VacA. VacA forms anion-selective channels, or pores, in cell or model membranes that share numerous properties with the host chloride, CLC, channels thus mimicking the characteristics of a host channel to conduct ions and perturb ion homeostasis in the stomach[104,105]. The VacA cytotoxin also promotes urea permeation in cultured MDCK, AGS, and Caco-2 cells[106] and was shown to enter cells, target mitochondria, and reduce mitochondrial membrane potential in a concentration-dependent manner[107]. Although it might be concluded that mitochondrial dysfunction would impact tight junction integrity by reducing ATP, urea permeation in the presence of VacA occurred by the transcellular, rather than paracellular, pathway and did not occur because of barrier dysfunction caused by damaging or otherwise reorganizing tight

junctions[106]. These studies further support the notion that VacA does not cause barrier dysfunction at tight junctions.

Urease and ammonia: In HGE-20 cell monolayers, luminal acid significantly increased TER and decreased paracellular permeability, which were affected by *H. pylori*, specifically in isogenic ureB$^-$ *H. pylori* mutants that produced considerable ammonia/ammonium[81]. This work suggested that *H. pylori* affects TER and permeability by neutralizing luminal acidity by the production of ammonia[66]. Although the claudin-expression in this cell line is largely unknown (Table 1), it is tempting to speculate that claudin 18 is modulated by luminal acidity in HGE-20 cells. In general, extracellular acidity stimulates cell signaling pathways, including extracellular signal-regulated kinase (ERK) and protein kinase C activation, which were demonstrated to regulate the expression of claudin 18 in MKN-45 cells but not in MKN74 or NUGC3 cells, which do not express claudin 18[108]. Following apical acidification, claudin 18 expression increased TER and reduced paracellular permeability when overexpressed in MDCK cells[35] further supporting the idea that ureB$^+$ *H. pylori* and ammonia may reduce tight junction function by modulating the expression of claudin 18. Although MKN28 cells do not express claudin 18 (Table 1), this cell line also demonstrated a significant decrement in TER with (Vac$^+$Cag$^+$) *H. pylori* that required ureB and ammonia/ammonium. However, this study concluded that barrier disruption was due to the activation of myosin light chain kinase[64]. Although the TER in Caco-2 cells exposed to (Vac$^+$Cag$^+$) *H. pylori* was dependent on the ammonia/ammonium-induced processing of occludin to a low molecular weight form[87], disruption of occludin does not cause barrier dysfunction in stomach, like it does in intestine[15], so it is likely that results with occludin are not relevant to stomach cells, *in vivo*.

CONCLUSION

In summary, tight junctions are configured slightly differently at the surface and in gastric glands but all claudin molecules are expressed in the mouse stomach with claudin 18 being the most prominent. While a comprehensive evaluation of claudin expression has not been done in the normal human stomach, human biopsy samples indicate that numerous claudins are expressed and that claudin 18 expression is also very high. These results suggest that stomach tight junctions are designed to be electrically tight and restrict cation permeability. Barrier dysfunction, including a reduction in TER and a significant increase in paracellular permeability, occurs *in vitro* and *in vivo* during *H. pylori* infection, consistent with a reduction in cation selectivity and an increase in the permeability of larger molecules due to significant changes in tight junction claudin expression and/or defects in tight junction integrity. When evaluating the role of *H. pylori* virulence factors in tight junction dysfunction, the most consistent results occur with urease and ammonia, which are thought to cause cytoskeletal rearrangement at tight junctions. The changes in claudin expression in human *H. pylori*-induced GC are inconsistent, making it difficult to predict molecular mechanisms that regulate tight junction dysfunction in patients. On one hand, claudin 18 expression is generally attenuated while the expression of other cation-limiting claudins increases, perhaps to compensate for the lack of permselectivity and barrier tightness in the absence of claudin-18. For the most part, studies in human patients choose either a single or subset of tight junction proteins to survey, but this strategy provides an inadequate snapshot of the total set of abnormalities that occur in a given patient tumor. It is possible that genetic variation in virulence factors associated with *H. pylori*, host genetic factors, and constitutive levels of inflammation result in variable results in population studies of claudin expression and its relevance to long-term survival. Furthermore, studies are required to determine whether or not claudin molecules have other roles in gastric cells, besides their classical role at tight junctions, to facilitate cancer development. Genome sequencing and immunostaining with concomitant cell culture studies done in appropriate models may assist with future endeavors to sort-out the role of *H. pylori* in barrier defects during infection.

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REVIEW

Signet-ring cell carcinoma of the stomach: Impact on prognosis and specific therapeutic challenge

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Abstract

While the incidence of gastric cancer has decreased worldwide in recent decades, the incidence of signet-ring cell carcinoma (SRCC) is rising. SRCC has a specific epidemiology and oncogenesis and has two forms: early gastric cancer, which can be resected endoscopically in some cases and which has a better outcome than non-SRCC, and advanced gastric cancer, which is generally thought to have a worse prognosis and lower chemosensitivity than non-SRCC. However, the prognosis of SRCC and its chemosensitivity with specific regimens are still controversial as SRCC is not specifically identified in most studies and its poor prognosis may be due to its more advanced stage. It therefore remains unclear if a specific therapeutic strategy is justified, as the benefit of perioperative chemotherapy and the value of taxane-based chemotherapy are unclear. In this review we analyze recent data on the epidemiology, oncogenesis, prognosis and specific therapeutic strategies in both early and advanced SRCC of the stomach and in hereditary diffuse gastric cancer.

Key words: Gastric cancer; Signet ring cell carcinoma; Diffuse gastric cancer; Hereditary diffuse gastric cancer; CDH1

Core tip: Contrary to others gastric cancer, the incidence of signet-ring cell carcinoma (SRCC) of the stomach is rising worldwide. SRCC has a specific epidemiology and oncogenesis and has two forms: early gastric cancer, which can be resected endoscopically in some cases and which has a better outcome than non-SRCC, and advanced gastric cancer, which is generally thought to have a worse prognosis and lower chemosensitivity than non-SRCC. Its poor prognosis may be due at least in part to its more advanced stage. Therapeutic

strategies are emerging but still controversial, as the benefit of perioperative chemotherapy and the value of taxane-based chemotherapy.

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INTRODUCTION

Gastric cancer (GC) is a major public health problem, with 951000 new cases identified worldwide in 2012, representing 6.8% of all new cases of cancers. During 2012, 723000 patients died of a gastric cancer, accounting for 8.8% of deaths from cancer[1]. GC is the fifth most frequently diagnosed cancer and the third leading cause of cancer-related death in the world. Despite a decrease in the overall incidence of gastric cancer in recent decades, the incidence of signet-ring cell carcinoma (SRCC) is constantly increasing, in Asia, the United States and Europe, accounting for 35% to 45% of gastric adenocarcinoma cases in recent studies[2,3]. Its incidence increased 10-fold between 1970 and 2000[4].

HETEROGENEITY OF PATHOLOGICAL CLASSIFICATIONS

This increase in the proportion of SRCC in cases of gastric adenocarcinoma can be explained by changes in the pathological classifications used to characterize these cancers. Since the publication of the WHO classification of gastric cancers in 1990, signet-ring cell adenocarcinoma constitutes one specific histotype and therefore can be better identified among gastric cancers. Previously, signet-ring cell adenocarcinoma was classified as "diffuse type" according to Lauren's classification[5], "infiltrative type" by Ming[6], "undifferentiated type" by Nakamura[7] and "high grade" by the UICC[8].

Now, signet-ring cell carcinoma is defined according to the WHO's classification as a poorly cohesive carcinoma composed predominantly of tumor cells with prominent cytoplasmic mucin and a crescent-shaped nucleus eccentrically placed[9] (Figure 1A). It is important to understand that signet-ring cell adenocarcinomas are always classified, by definition, as "undifferentiated type" by Nakamura and as "diffuse type" by Lauren. But, conversely, not all gastric cancers classified as "undifferentiated" or "diffuse" are signet-ring cell cancers.

Also, although it is the usual histotype of linitis plastica, signet-ring cell adenocarcinoma should be distinguished from linitis plastica, which is defined macroscopically by thickening and rigidity of the gastric walls secondary to an abundant fibrous stromal reaction (Figure 1B). Thus 10% to 20% of cases of linitis plastica are not due to signet-ring cell adenocarcinoma[10].

EPIDEMIOLOGY OF SRCC:

Unlike non-SRCC, the incidence of SRCC of the stomach is rising

Since the advent of treatment to eradicate Helicobacter, the incidence of gastric adenocarcinoma has decreased. However, the incidence of SRCC is rising and SRCC is found in 8% to 30% of gastric cancers. SRCC epidemiology and risk factors differ substantially from those of other types of gastric adenocarcinoma. SRCC is more frequent in women than non-SRCC, with a sex ratio around 1, compared with less than 1/2 in gastric adenocarcinoma. SRCC occurs in younger patients, consistently 7 years before non-SRCC, with a mean age ranging from 55 to 61 years[3,11]. Ethnic distribution is unclear. A previous report showed a lower frequency in Asians, but SRCC as a disease entity was not clearly separated[9]. In a recent study in more than 10000 patients with gastric cancer, SRCC was significantly more common among black, Asian/Pacific Islander, American Indian/Alaska Native, and Hispanic ethnic groups[3]. In particular, in the Asian population, which represented 14% of the total population in this study, which is quite low considering the known epidemiology of gastric cancer in Asians, SRCC was found in more than 30% of patients. Another study on 1884 patients with less than 10% of Asian patients gave the same results[12]. But these studies were conducted in the United States and Canada and Asian patients living in North America may not be representative of the global Asian population. However, in recent large study in Asian countries SRCC was found in 15% of patients in South Korea[11], in 10% of Japanese patients[13] and in 6% to 15% of patients in China[14,15], although recent studies from the United States or European countries show a frequency of 25% to 30%[3,10].

SRCC has a distinct clinical presentation from non-SRCC

Considering clinical presentation, SRCC is more frequent in the middle stomach than non-SRCC. SRCC type is associated with more advanced cancer and is most frequent in stage 4, T3/T4 and N2 cancers. Paradoxically, SRCC is more frequent in early gastric cancer than in advanced gastric cancer in some reports[11]. In fact, SRCC in early gastric cancer and advanced gastric cancer may represent 2 distinct subsets with distinct implications. In advanced gastric cancer, peritoneal carcinomatosis is the most frequent metastatic site[16], and some authors recommend

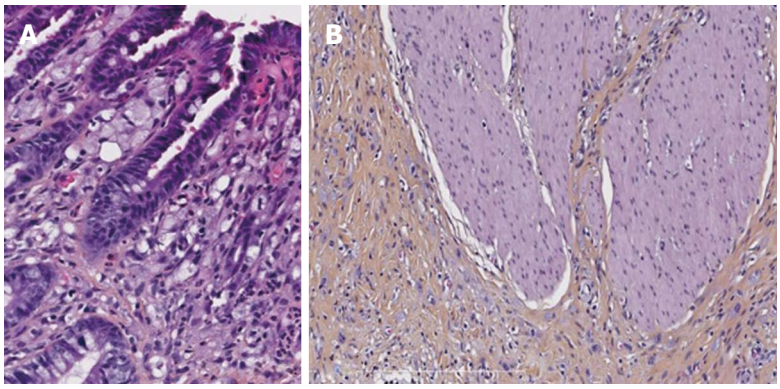

Figure 1 Focus of intramucosal signet ring cell carcinoma invading the lamina propria (T1a) (A) and signet ring cell carcinoma invading muscularis propria as single tumor cells with marked desmoplasia (B).

routine laparoscopic evaluation before treatment.

SRCC shares no risk factors with non-SRCC

In accordance with the different epidemiologies, SRCC could have different risk factors from non-SRCC. While non-SRCC is often multifactorial, infection with *Helicobacter pylori* (*H. pylori*) leading to chronic gastritis is involved in most cases of gastric cancer, with the exception of cardia cancer. However, the role of *H. pylori* in SRCC is more controversial. Indeed, since wide eradication of *H. pylori*, an *H. pylori*-negative gastric cancer (*H. pylori*NGC) entity has been emerging. This entity may include several subtypes, such as gastric adenocarcinoma of the fundic gland ((GA-FG-CCP) and SRCC, thus questioning the role of *H. pylori* in these histologic subtypes[17].

The role of other risk factors in gastric cancer (salt-preserved food, smoking, auto-immune gastritis) or cardia cancer (obesity…) is not well studied in SRCC.

SRCC is associated with specific germline mutations in the CDH1 gene, which encodes the epithelial cell adhesion protein E-cadherin in patients with hereditary diffuse gastric cancer

Early-onset diffuse gastric cancer (DGC), multi-generational DGC and lobular breast cancer clinically define hereditary diffuse gastric cancer (HDGC). Updated criteria were established by a multidisciplinary workshop in 2015[18].

CDH1 germline mutations are the main genetic cause of HDGC. The first CDH1 germline mutation was described in 1998, with a founder mutation identified in the New Zealand Maori population[19]. A heterozygous CDH1 germline mutation increases the lifetime of DGC and lobular breast cancer.

In the updated recommendations, compared with the 2010 guidelines[20], in the case of a familial history of gastric cancer the age of diagnosis is no longer required, as soon as DGC is confirmed histologically for at least one case. Two groups have been added in families in whom genetic testing can be considered: individuals with a personal or family history of cleft lip/cleft palate and DGC; *in situ* signet-ring cells and/or pagetoid spread of signet-ring cells in the stomach. The revised criteria are summarized in Table 1.

Using the 2010 criteria, the CDH1 detection rate is between 10% and 18% in countries with a low incidence. In contrast, this detection rate is much higher in the New Zealand Maori population[21-23]. A recent study updated penetrance data for CDH1 mutations carriers from 75 families. By the age of 80 years, the cumulative risk of DGC is estimated to be 70% for men (95%CI: 59%-80%) and 56% for women (95%CI: 44%-69%). Moreover, the cumulative risk of lobular breast cancer is reported to be 42% (95%CI: 23%-68%). No evidence for an increased risk of other types of cancer has been noted[21].

Within pathogenic CDH1 germline mutations, there is a majority of truncating mutations that do not lead to a functional protein. Rare large exonic deletions exist, with a frequency of about 5%[24]. As CDH1 is a tumor suppressor gene, a second somatic hit is needed for tumor initiation, which most frequently includes promoter methylation, and less frequently somatic mutation or loss of heterozygosity[25].

Other genes can be considered as candidates in HDGC predisposition: CTNN1A, BRCA2, PALB2 and MAP3K6. So far, no recommendation can be offered, due to lack of data[21,26].

CDH1 germline mutation carriers should be strongly advised to undergo prophylactic total gastrectomy, usually between 20 and 30 years old. Family history should be taken into account, especially the age of onset of clinical cancer in probands. Baseline endoscopy should be performed before surgery and *H. pylori* infection should be screened for and infected patients should be excluded. Gastrectomy examination and sampling should follow a specific protocol. Nearly all samples harbor signet-ring cells and many harbor T1a carcinoma[27].

Annual endoscopy should be offered to subjects who do not undergo surgery. To this end, a white light high-definition endoscope is recommended, for a least 30 min, with repeated inflation and deflation, in order

Table 1 Clinical hereditary diffuse gastric cancer testing criteria (from van der Post J Med Genet 2015[18])

Criteria include first and second degree relatives	
Established criteria	2 GC cases regardless of age, at least one confirmed DGC One case of DGC < 40 Personal or family history of DGC and LBC, one diagnosed < 50
Families in whom testing could be considered	Bilateral LBC or family history of 2 or more cases of LBC < 50 Personal or familial history of cleftlip/palate in a patient with DGC *In situ* signet ring cell and/or pagetoid spread of signet ring cells

GC: Gastric cancer; DGC: Diffuse gastric cancer; LBC: Lobular breast cancer.

to inspect the mucosa carefully. A minimum of 30 biopsies is recommended. Any endoscopically visible lesions are biopsied, including pale areas, but random sampling should also be performed, five biopsies being taken from each of the following anatomical zones: pre-pyloric area, antrum, transitional zone, body, fundus and cardia.

In women with a CDH1 mutation, breast surveillance includes annual breast magnetic resonance imaging (to which mammography can be added) starting at the age of 30, combined with an annual clinical breast examination. Prophylactic mastectomy is not recommended, but can be considered for some women.

There is no evidence to link CDH1 mutation to an increased risk of colorectal cancer, but case reports have mentioned colorectal and appendiceal SRCC in CDH1 mutation carriers. Therefore, in CDH1 mutation families in which colon cancer is reported in mutation carriers, colonoscopy screening can be proposed at age 40 or 10 years younger than the youngest diagnosis of colon cancer, whichever is younger, and repeated at intervals of 3-5 years[18].

SPECIFIC PATHWAYS ARE IMPLICATED IN SRCC CARCINOGENESIS

SRCC has a specific oncogenesis that differs from that of tubular gastric adenocarcinoma. The two main pathologic processes at a cellular level are loss of cell-cell adhesion molecules and accumulation of mucin in large vacuoles.

E-cadherin, which is encoded by the *CDH1* gene, is a cell-cell adhesion molecule and seems to play a key role in carcinogenesis. Its role in tumor progression and epithelial-mesenchymal transition has been widely studied in many types of cancer[28,29], but in SRCC E-cadherin may be involved earlier in tumor initiation[30]. E-cadherin deficiency has been reported to initiate carcinogenesis in a large proportion of SRCC cases, in both HDGC and sporadic SRCC. As seen above, germline inactivating truncating mutations in CDH1 are found in some, but not all, cases of HDGC[31]. These mutations confer an autosomal dominant susceptibility with variable penetrance according to the family. The carcinogenesis model in HDGC supposes that in patients carrying the germline mutation, a somatic event could occur in the second allele, such as a point mutation, loss of heterozygosity, or more frequently promoter hypermethylation[32]. Host-environment interaction could play a role in this somatic mutation (diet, gastritis, carcinogens)[30,31,33-35]. It is of note that CDH1 mutations are not found in familial intestinal gastric adenocarcinoma.

In sporadic SRCC, somatic mutations of CDH1 are also frequently involved compared with gastric adenocarcinoma, mostly promoter hypermethylation[36].

While CDH1 mutations seem to be the most frequent abnormality leading to SRCC, other adherence molecules could be involved in fewer cases, such as somatic mutations of β-catenin/APC genes or dysregulation of the Wnt/β-catenin pathway[37].

Moreover, expression of CDH1 and other adherence molecules could be downregulated upstream of various pathways. The phosphatidylinositol 3-kinase (PI3K) pathway may be involved in some cases of SRCC carcinogenesis. Briefly, the activated ErbB2/ErbB3 complex in SRCC binds PI3K leading to phosphorylation of tyrosine residues and activation of downstream pathways including p38 MAP kinase. Activation of p38 MAP kinase lead to loss of cell-cell contact by disruption of adherent junctions[38]. Moreover, the MEK1 pathway may complete the loss of cell-cell contact, and other pathways, as yet not well described, are probably involved. MUC4 has been reported to increase activation of the ErbB2/ErbB3 complex. MUC4 belongs to the family of mucins that are normally expressed in gastric mucosae (MUC1, MUC5AC, MUC6) or expressed *de novo* in gastric cancer (MUC2, MUC4). In SRCC, accumulation of mucins results in large vacuoles, which could therefore play a role in carcinogenesis. However, the mechanisms and pathways underlying mucin secretion and accumulation in cells are not well known.

Finally, a hormonal theory in which estrogen is involved in tumor initiation or progression or both has been developed to explain the increased incidence in women of SRCC compared with non-SRCC. Indeed, diffuse type gastric cancer is more likely to present estrogen receptors, even if this is not well established in the SRCC subtype[39-41]. However, while this mechanism has been suggested to be involved in the tumor process, there is no evidence that it plays a major role.

PROGNOSIS OF SIGNET-RING CELL GASTRIC ADENOCARCINOMA

While all studies agree on the poor prognosis of

Table 2 Studies assessing prognosis of the signet-ring cell histotype in early gastric cancers

Ref.	Number of patients in study	Number of early gastric cancers	SRCC frequency in early gastric cancer	Prognosis of SRCC (type of analysis)
Maehara *et al*[57] (1992)	1500	384	7.3%	Similar (univariate)
Otsuji *et al*[58] (1998)	1498	568	19.8%	Better (univariate)
Hyung *et al*[80] (2002)	3104	933	28.2%	Better (univariate)
Kim *et al*[45] (2004)	2358	561	16.7%	Similar (multivariate)
Kunisaki *et al*[43] (2004)	1113	513	23.4%	Better (multivariate)
Ha *et al*[42] (2008)	1520	1520	25.5%	Better (univariate)
Jiang *et al*[44] (2011)	2315	269	20.1%	Better (multivariate)
Kwon *et al*[11] (2014)	769	326	15.6%	Better (multivariate)
Gronnier *et al*[46] (2013)	421	421	25%	Similar (multivariate)

SRCC: Signet-ring cell carcinoma.

diffuse gastric adenocarcinoma according to Lauren's classification, including SRCC, the prognosis of signet-ring cell adenocarcinoma is still debated and appears to depend on the stage of the cancer at the time of diagnosis.

Prognosis of signet ring cell adenocarcinoma in early gastric cancers

For early gastric cancer, described by the Japanese Endoscopy Society as gastric cancer not extending beyond the submucosa whatever the lymph node status, the prognosis of SRCC has been reported in all studies as equivalent to or better than that of other gastric adenocarcinomas. Thus, in the largest published study of early gastric cancer in 1520 patients which compared prognosis of SRCC and non-SRCC, patients with SRCC had a better survival rate than patients with other gastric adenocarcinomas[42]. Among the nine studies that specifically studied the prognostic impact of the histotype (SRCC or non-SRCC) in early gastric cancers, five conducted a multivariate analysis to take account of potential confounding factors (Table 2). Three studies demonstrated that survival was better in early SRCC than in other early gastric cancers (Kunisaki *et al*[43] HR = 0.28; 95%CI: 0.08-0.91)[11,44] and two studies showed that the prognosis was similar[45,46].

This better overall survival observed in most studies could be related to the younger age at presentation for SRCC patients, as suggested by Gronnier *et al*[46]. Moreover, SRCC was more frequently limited to the mucosa and had fewer invaded lymph nodes than non-SRCC in early gastric cancer, which are two well-known prognostic factors for survival.

Prognosis of signet-ring cell adenocarcinoma in advanced gastric cancer

Conversely, in advanced gastric cancer, the prognosis of signet-ring cell adenocarcinoma is more controversial and is commonly thought to be poor. This was first suggested in retrospective studies[47-52], without distinction of SRCC among diffuse types. Two retrospective studies of more than 3500 patients with advanced SRCC showed a significantly worse 5-year survival rate than in non-SRCC[53,54] (Table 3). Other smaller studies showed a significant difference in overall survival between differentiated, SRCC and undifferentiated gastric cancer, SRCC being close to undifferentiated[11,14,55]. But other small studies did not indicate a significantly worse prognosis of SRCC[43-45,56-58]. Another study showed that SRCC was an independent predictor of poor prognosis in multivariate analysis[10], though this was not significant in another study with multivariate analysis[59]. Most of these studies were Asian.

Finally, the largest cohort comparing SRCC and non-SRCC, in more than 10000 patients, did not report that SRCC was a prognostic factor after adjustment for the tumor stage in advanced gastric cancer. However, Taghavi *et al*[3] did not specify the precise percentage of SRCC cases and did not use the WHO classification for more than 50% of SRCC cases. In this cohort, SRCC was not predictive of poor outcome, but was associated with more aggressive tumors. SRCC was more likely to be associated with an American Joint Committee on Cancer stage 4 tumor (50% *vs* 42%, $P < 0.001$), T3/T4 tumor (45.8% *vs* 33.3%, $P < 0.001$) and N2/N3 tumor (59.7% *vs* 51.8%). But in this large registry cohort, some confounding clinicopathological factors were not known, such as Performance Status, type of resection, and perioperative treatment. Moreover, it is quite surprising that patients with SRCC at a more advanced stage did not have a worse prognosis in univariate analysis. So, even though the size of the cohort is impressive, these data do not close the debate.

In conclusion, the prognosis of SRCC in advanced gastric cancer is controversial. Some reports suggest a worse prognosis, while others suggest that the presence of SRCC in gastric adenocarcinoma is not an independent predictor of prognosis after adjustment for the stage. But in most studies, SRCC was at a more advanced stage, suggesting a more aggressive SRCC phenotype and lower R0 resection rate[55], which could explain the poorer prognosis in some studies. This hypothesis is supported by results from several studies in which SRCC had a worse prognosis univariate analysis, but not in multivariate analysis, after adjustment for the tumor stage[14,45,54].

Table 3 Studies assessing the prognosis of the signet-ring cell histotype in advanced gastric cancer

Ref.	Number of patients in study	Number of advanced gastric cancers	SRCC frequency in advanced gastric cancer	Median 5-yr survival of SRCC (*vs* non-SRCC)	*P*-value
Maehera *et al*[57] (1992)	1500	1116	2%	48% (*vs* 33%)	NS
Kim *et al*[53] (1994)	3702	NP	NP	32% (*vs* 45%)	< 0.05
Otsuji *et al*[58] (1998)	1498	630	9.5%	44% (*vs* 28%)	NS
Yokota *et al*[56] (1998)	923	NP	NP	11% (*vs* 38%)	NS
Theuer *et al*[59] (1999)	3020	NP	NP	NP	NS (multivariate)
Kim *et al*[45] (2004)	2358	1797	6%	35% (*vs* 40%)	NS
Kunisaki *et al*[43] (2004)	1113	600	9%	NP	NS
Li *et al*[42] (2007)	4759	4759	14%	42% (*vs* 51%)	0.009
Messager *et al*[68] (2011)	159	NP	NP	9% (*vs* 24%)	0.038
Taghavi *et al*[3] (2013)	12246	6261	26.3%	NP	NS (multivariate)
Jiang *et al*[44] (2011)	2315	2046	7%	31.5% (*vs* 35.7%)	NS
Kwon *et al*[11] (2014)	769	443	12.8%	26% *vs* 50.5%[1]	0.004
Zu *et al*[14] (2014)	741	741	5.9%	43.4% *vs* 87.1%[2]	0.012[3]
Heger *et al*[55] (2014)	723	312	33.5%	NP	0.02 (multivariate)

[1]Ten-year survival; [2]*vs* well-differentiated cancer; [3]Comparison between all histotypes (well differentiated, moderately differentiated, poorly differentiated and SRCC). SRCC: Signet-ring cell carcinoma.

THERAPEUTIC STRATEGIES

Early gastric cancer: How far can we perform endoscopic resection?

The presence of lymph node metastases is considered as one of the most significant prognostic factors for overall and disease-free survival in patients with gastric cancer. Therefore, it is essential to highlight this potential lymph node involvement with appropriate surgery and consequently with extended lymphadenectomy, but also to propose postoperative chemotherapy when indicated.

However, for some early gastric cancers, the risk of lymph node metastasis is thought to be very low. Thus, patients with a well to moderately well differentiated tumor of less than 3 cm in size without submucosal invasion as well as patients with a well-differentiated, nonulcerated and limited submucosal lesion (T1sm1) of less than 3 cm in size have no risk of lymph node metastasis according to Gotoda *et al.* In these cases, endoscopic treatment including endoscopic mucosal resection or endoscopic submucosal dissection can be an alternative to radical surgery and has better perioperative outcomes and comparable long-term results[60,61].

Conversely, patients with early gastric cancer limited to the mucosa (clinically T1a), but with an ulcerated lesion, a lesion larger than 3 cm, with undifferentiated histotype or with lymphatic duct invasion have an increased risk of lymph node metastasis (detailed in Table 4). For these reasons, various guidelines have been established to define the indications for endoscopic resection. In Asia, endoscopic mucosal resections are limited to well or moderately differentiated tumors of less than 2 cm in size, limited to the mucosa and non-ulcerated, according to the Japan Gastric Cancer Association (JGCA) guidelines. Moreover, endoscopic submucosal resection, which enables more complete and extensive *en-bloc* resection, is indicated by JGCA guidelines for well-differentiated and non-ulcerated tumors of more than 2 cm in size and extending up to the submucosa (sm1) or for well-differentiated and ulcerated tumors of less than 3 cm limited to the mucosa or for undifferentiated and non-ulcerated tumors of less than 2 cm limited to the mucosa (Table 4).

In Europe and the United States, the EORTC St. Gallen International Expert Consensus defines the indications for endoscopic resections of early gastric cancer, largely following JGCA guidelines, except for gastric cancers with diffuse histology for which surgery is considered obligatory[62]. Thus, it is not recommended to perform endoscopic resection for early signet-ring cell gastric cancer in western countries, whatever the depth of invasion in the gastric walls. In Asia, SRCC limited to the mucosa, non-ulcerated and less than 2 cm in size can be resected by submucosal endoscopic resection[63]. In a recent study, Ha *et al*[42] supported this indication by demonstrating no lymph node metastasis in 77 patients with early gastric cancer confined to the mucosa, less than 2 cm in size and with no lymphatic involvement.

Resectable gastric cancers: Which procedure for signet-ring cell carcinoma?

For non-metastatic advanced gastric cancer, endoscopic resection is not possible due to a too high risk of lymph node metastases. Surgical resection is then essential to treat these tumors, combined with an adequate lymphadenectomy in order to assess the patient's prognosis, avoid stage migration and to propose the most appropriate therapeutic strategy.

The extent of this lymphadenectomy during gastrectomy for resectable advanced gastric cancer has been debated between Western and Asian surgeons for long time. Thus, despite a theoretical advantage of offering the widest lymphadenectomy possible, as advocated by Asian surgeons, two

Table 4 Incidence of lymph node metastasis in early gastric cancer (according with Gotoda *et al*[81])

Depth of invasion	Tumor size	Grade of differentiation	Ulcerated versus not ulcerated tumor	Incidence of LNM	Recommended treatment
Mucosal	< 2 cm	Well differentiated	Not ulcerated	0%	EMR
		Poorly differentiated	Not ulcerated	0%	ESD (Asia)/surgery (Western)
		Well differentiated	Ulcerated	0%	ESD
		Poorly differentiated	Ulcerated	2%	Surgery
	2-3 cm	Well differentiated	Not ulcerated	0%	ESD
		Poorly differentiated	Not ulcerated	1.7%	Surgery
		Well differentiated	Ulcerated	0%	ESD
		Poorly differentiated	Ulcerated	2.4%	Surgery
	> 4 cm	Well differentiated		1.7%	Surgery
		Poorly differentiated		7.3%	Surgery
Submucosal (sm1)	< 3 cm	Well differentiated		5.6%	ESD/Surgery
		Poorly differentiated		NC	Surgery
	> 3 cm	Well differentiated		2.6%	Surgery
		Poorly differentiated		6.5%	Surgery
Submucosal (sm2)	< 3 cm	Well differentiated		19%	Surgery
		Poorly differentiated		NC	Surgery
	> 3 cm	Well differentiated		27%	Surgery
		Poorly differentiated		NC	Surgery

ESD: Endoscopic submucosal dissection.

controlled randomized trials comparing D1 *vs* D2 lymph node dissection have demonstrated no 5-years survival benefit and higher postoperative mortality for D2 lymphadenectomy[64,65]. Nevertheless, both trials have received criticism over the relative inexperience of many different surgeons performing D2 lymphadenectomy, which could explain the higher mortality observed in D2 lymphadenectomy group. Furthermore after a follow-up of 15 years, D2 lymphadenectomy was associated with lower locoregional recurrence and gastric cancer-related death rates than D1 surgery in the Dutch D1D2 trial[66]. Thus, to deal with this lower locoregional recurrence rate associated with higher postoperative morbidity and mortality rates linked to splenectomy and distal pancreatectomy, a modified D2 lymphadenectomy (without splenectomy and distal pancreatectomy, named also D1,5 lymphadenectomy) was proposed, and become the standard lymphadenectomy for advanced gastric cancer in some European countries as in France, whereas the D2 lymphadenectomy remains the standard in others.

Despite a higher rate of lymph node involvement in SRCC, no specific recommendation is available about the type of lymphadenectomy to perform for advanced SRCC. As for other histological types, a modified D2 lymphadenectomy to remove at least 15 lymph nodes is recommended.

For distal gastric cancer, only two randomized clinical trials have investigated whether subtotal gastrectomy is sufficient compared with total gastrectomy. Both trials indicated no statistical difference in mortality or survival between the two surgical procedures. No subgroup analysis was conducted to evaluate these two procedures based on histological type. Thus, subtotal gastrectomy is recommended for antro-pyloric cancer, whatever the histological subtype. However, because the infiltrative nature of the SRCC results in more frequently invaded proximal and distal resection margins (20.3% *vs* 9.0% and 20.3% *vs* 4.0% in Piessen *et al*[10]), some authors routinely perform total gastrectomy combined with freezing of resection margins in the case of antro-pyloric SRCC.

Finally, due to a high rate of peritoneal carcinomatosis (17%) discovered during surgical resection of advanced SRCC, certain surgeons propose two specific therapeutic strategies for SRCC. First, staging laparoscopy can be performed routinely before any treatment to track any peritoneal carcinomatosis and therefore to modify treatment. Second, in the event of intraoperative discovery of resectable peritoneal carcinomatosis, palliative resection is not recommended for advanced SRCC because of an unacceptable three-fold higher risk of postoperative mortality for this histological subtype[67].

SRCC may have a different chemosensitivity profile than non-SRCC

SRCC is thought to be less chemosensitive than non-SRCC. However, no specific studies have assessed this hypothesis, which is supported by several controversial findings.

In a retrospective study of 924 cases of resected SRCC, comparing patients with and without peri-operative chemotherapy, the latter provided no benefit in terms of R0 resection rate (about 65%) or in survival[68]. Morever, perioperative chemotherapy was found to be an independent predictor of poor survival (HR = 1.4, 95%CI: 1.1-1.9, P = 0.042) and the authors suggested as an explanation that toxicity of neoadjuvant treatment was correlated with worse outcome[69]. However, this study suffers from several biases. The indication for perioperative treatment was

left to the investigator. Patients receiving perioperative chemotherapy had a more aggressive presentation than patients who received no perioperative treatment. Furthermore, the type of chemotherapy was left to the choice of the investigator. Perioperative standards are based on mostly non-SRCC or nonspecific studies and most patients receive 5FU + platinum component +/- epirubicin. Conversely, another large retrospective study in a perioperative setting suggested that SRCC has a lower response rate to neoadjuvant chemotherapy (mostly 5FU + platinum), but either the clinical or pathological response was significantly correlated with a better outcome[55]. This result highlights that perioperative treatment in SRCC may confer a theoretical benefit, but that the classic regimen seems insufficient.

SRCC could have a different chemosensitivity profile, and in particular recent data suggest that taxane-based therapy could be more efficient in SRCC. An ex vivo analysis of chemosensitivity of several human gastric cancer samples showed that SRCC and diffuse-type samples were significantly more sensitive to such drugs as mitomycin C, doxorubicin and docetaxel than intestinal-type samples, but not to 5FU or platinum[70], which is still most often used in the perioperative setting. In a comparison of docetaxel- and oxaliplatin-based chemotherapy in various SRCC histologies, Chen *et al*[71] found a benefit of docetaxel-based chemotherapy in mixed SRCC. However, the results were conflicting in pure SRCC in which there was no difference between the two types of chemotherapy. In a retrospective study with a limited number of patients (n = 17), docetaxel-based chemotherapy was associated with an 80% R0 resection rate and a median overall survival of more than 40 mo[72].

In a metastatic setting there are few data concerning chemosensitivity in specific subsets of SRCC in prospective trials. Twenty years ago Rougier *et al*[73] reported a 16% response rate in SRCC compared with 65% in non-SRCC. However, in a metastatic setting also, drugs such as taxanes may be more effective. We reported that in diffuse type SRCC and in SRCC patients treated with docetaxel, the combination of 5FU and oxaliplatin gave a response rate of more than 65% and seemed at least equivalent in non-SRCC[74,75].

Specific oncogenic pathways may induce specific sensitivity to targeted agents. There are no data concerning SRCC in recent trials testing targeted agents in gastric cancer. However, efficacy in diffuse type has been studied in a few trials. In the REGARDS trial, which was a phase Ⅲ trial testing ramucirumab, an anti-VEGFR2 antibody, versus best supportive care in pretreated patients with gastric cancer, ramucirumab provided a significant benefit in overall survival[76]. In subgroup analysis, a high benefit was found in the diffuse type (HR = 0.56; 95%CI: 0.36-0.85), but not in the intestinal type, suggesting higher sensitivity to antiangiogenics. This was not found in the RAINBOW trial testing ramucirumab in combination with paclitaxel[77], or with targeted therapy including anti-HER2, which is validated in HER2-overexpressing gastric cancer[78]. However, diffuse type was a small subgroup in these trials, and so we cannot draw conclusions regarding specific sensitivity.

Finally, immunotherapy should be tested in SRCC, as PDL1 is overexpressed in about 23% of cases of SRCC, and anti-PDL1 antibody is a promising treatment of GC[79].

In conclusion, whereas SRCC is thought to be less chemosensitive than non-SRCC, recent reports suggest it could have a specific sensitivity profile and be more sensitive to taxane-based chemotherapy or antiangiogenics. However, this has to be confirmed in a specific prospective trial. In a perioperative setting, the benefit of chemotherapy is controversial and a prospective randomized trial is under way to test this hypothesis. However, the chemotherapy regimen used is the old combination of epirubicin, cisplatin and fluorouracil, which may not be the optimal regimen in SRCC.

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REVIEW

Aspects of the non-pharmacological treatment of irritable bowel syndrome

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Abstract

Irritable bowel syndrome (IBS) is one of the most commonly diagnosed gastrointestinal conditions. It represents a significant healthcare burden and remains a clinical challenge. Over the years IBS has been described from a variety of different perspectives; from a strict illness of the gastrointestinal tract (medical model) to a more complex multi-symptomatic disorder of the brain-gut axis (biopsychosocial/psychosomatic model). In this article we present aspects of the pathophysiology and the non-pharmacological treatment of IBS based on current knowledge. Effects of conditioned stress and/or traumatic influences on the emotional system (top-down) as well as effects on the intestine through stressors, infection, inflammation, food and dysbiosis (bottom-up) can affect brain-gut communication and result in dysregulation of the autonomic nervous system (ANS), playing an important role in the pathophysiology of IBS. Conditioned stress together with dysregulation of the autonomic nervous system and the emotional system may involve reactions in which the distress inside the body is not recognized due to low body awareness. This may explain why patients have difficulty identifying their symptoms despite dysfunction in muscle tension, movement patterns, and posture and biochemical functions in addition to gastrointestinal symptoms. IBS shares many features with other idiopathic conditions, such as fibromyalgia, chronic fatigue syndrome and somatoform disorders. The key to effective treatment is a thorough examination, including a gastroenterological examination to exclude other diseases along with an assessment of body awareness by a body-mind therapist. The literature suggests that early interdisciplinary diagnostic co-operation between gastroenterologists and body-mind therapists is necessary. Re-establishing balance in the ANS is an important component of IBS treatment. This article discusses the current knowledge of body-mind treatment, addressing the topic from a practical point of view.

Key words: Irritable bowel syndrome; Assessment; Treatment; Hypnotherapy; Pathophysiology; Body awareness therapy; Psychosomatics; Stress; Body-

mind

Core tip: Due to the complex nature of irritable bowel syndrome (IBS), no long-lasting generally accepted therapies are available. Different lines of research have been developed to address this issue. One line focuses on identifying intestinal mechanisms that may be affected by pharmacologic intervention. The understanding of IBS, especially the interactions between the central and enteric nervous systems, has grown considerably in recent years. Because recent research has focused more on the body-mind aspect of the disease, body-mind remedies such as hypnotherapy, psychotherapy and body awareness therapy have been applied. In highlighting this topic we discuss non-pharmacological methods and practical guidelines for the treatment of IBS.

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INTRODUCTION

Irritable bowel syndrome (IBS) is one of the most commonly diagnosed gastrointestinal conditions, characterized by symptoms such as abdominal pain, cramping or abdominal bloating, faecal urgency, and alteration of bowel habits with the relief of pain or discomfort upon defecation. Women are more frequently diagnosed with IBS than men. IBS patients are generally subdivided into diarrhoea predominant (D-IBS), constipation predominant (C-IBS) or an alternating type (A-IBS), which stool fluctuates between diarrhoea and constipation[1-5].

IBS generates a significant healthcare burden[4] with huge economic costs[6-9]. Increased economic consequences are also incurred as a result of unnecessary surgery. A threefold higher rate of cholecystectomy, a twofold higher rate of appendectomy and hysterectomy and an approximately 50% higher rate of back surgery have been recorded in IBS patients compared to those without IBS[4,10]. The severity of symptoms varies widely, from very mild to incapacitating. The prevalence of moderate and severe cases may be underestimated[11]. Previous studies have highlighted how IBS impairs health-related quality of life, possibly even increasing the risk for suicidal behaviours[12,13]. An IBS diagnosis is based on clinical symptoms and the exclusion of somatic diseases[14,15]. Clinical symptoms have often been defined through questionnaires including the Manning, Kruis Score, Rome Criteria, Abdominal Symptom Questionnaire and the Gastro Intestinal Scale[15-19]. These questionnaires differ in how the questions are formulated. To be classified as IBS according to Rome Ⅱ, patients answer, "yes or no" to the question; "in the last 3 mo, did you often have discomfort or pain in your abdomen?" If they answer "no" they do not have IBS. While in the Gastro Intestinal Scale, the questions consist of a seven-point scale from no discomfort to the worst conceivable symptoms. In the Rome Ⅲ questionnaire more alternatives in most of the questions are provided[18]. In our experience, individuals rate their pain in different ways depending on their earlier life experiences, body awareness, gender, *etc*.

In addition to gastrointestinal symptoms, IBS patients often experience a wide range of other problems, such as non-abdominal pain, psychological symptoms, low quality of life, as well as difficulties in carrying out activities of daily life[13,20,21]. They also exhibit complicated body tensions, bodily stress patterns, low body awareness and abnormal stress parameters[18,22-27]. Many IBS patients have been exposed to traumatic events and may also have low self-esteem, difficulties setting limits and hypersensitivity[28,29]. Therefore IBS patients may show many signs of being in a state of chronic distress.

IBS over the years

In 1948, Collins defined the syndrome of irritable colon as a hyperirritable, neuromuscular imbalance of the colon sufficiently severe to cause abdominal pain or distress[30]. He stated his long-time interest in the dysfunction of the gastrointestinal tract due to functional as well as somatic causes: "The purpose of this communication is to emphasize physiologic, local irritative and psychosomatic factors"[30]. In 1956, Bargen[31] wrote, "The so called irritable colon is primarily the result of an emotional disturbance, a tension state, abuse of laxative agents or a dietary indiscretion" and concluded in his article that "actually, there are no medicines that are substituted for a carefully planned program of management of the digestive problems of these persons. Measures should include particular attention to their emotional disturbances, their situation in respect to stress, and particularly their dietary problems".

During the sixties, IBS was defined as a disease of the gastrointestinal region and treatment was largely pharmacological. In 1999, Wessely *et al*[32] wrote an article entitled "Functional somatic syndromes: one or many?", after which several physicians expressed their frustration about the management of IBS. Enck *et al*[33] wrote in 2008, "the next consensus for the irritable bowel syndrome has to be interdisciplinary".

In the late seventies the term "biopsychosocial" was introduced; since then, over 90 articles have been published according to PubMed using this term

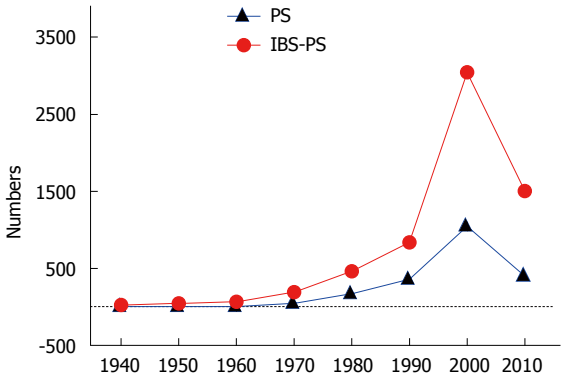

Figure 1 Number of references in psychosomatic journals and in medical-gastroenterological journals from 1940 to 2012, presented in decades. PS: Psychosomatic journals; IBS-PS: Medical-gastroenterological journals.

in reference to IBS. Throughout the years, IBS articles have been published in both psychosomatic journals as well as in medical-gastroenterological journals (Figure 1). The search terms used were IBS/irritable bowel*/psychosomatic*(PS) and IBS/irritable bowel*-psychosomatic* (IBS-PS), published from 1940-1949 through 2010-2012. The number of articles from medical-gastroenterological journals outnumbers those from psychosomatic journals. Throughout the literature, two views emerge; one is the medical view of IBS as a strict disease of the gastrointestinal tract, while the other is the psychosomatic/biopsychosocial view in which IBS is seen as a more complex multi-symptomatic disorder. Ålander and others suggest that IBS causes an increased demand on healthcare due to increased overall co-morbidity, thus requiring a more holistic approach to understand the underlying mechanisms and develop effective treatments[21,29,34].

Associated conditions

Substantial evidence exists that IBS shares many features with other syndromes such as fibromyalgia, chronic fatigue syndrome, somatoform disorders, and unexplained urological conditions[35,36]. The diagnosis given to a patient with one of these conditions often depends on the hallmark symptom and the expertise of the treating clinician rather than on the condition itself[32,35].

The syndromes above have also been called functional somatic syndromes, medically unexplained symptoms, somatoform disorders or unexplained clinical conditions. These syndromes are often characterized by a lack of a clear physical or biological aetiology or an inconsistent demonstration of laboratory abnormalities[32,35,36]. It has been suggested that these conditions should be gathered under one common name, such as bodily distress syndrome, central sensitivity syndrome, or dysfunctional syndrome[36,37]. These patients are likely to consult primary health care as well as different specialty departments at the hospital.

PATHOPHYSIOLOGY

Two directions

Within the past decade there has been increasing evidence supporting the concept of IBS as a multi-symptomatic disorder of brain-gut function[35,38,39]. The brain and the enteric nervous system communicate through the autonomic nervous system (ANS) and the hypothalamic-pituitary-gut axis[40]. This communication allows stressors in the brain to influence gut function (top-down) and stressors in the gut to influence the brain (bottom-up). This bidirectional signalling can result in the dysregulation of the autonomic nervous system, which may play an important role in the pathophysiology of IBS[41,42].

Top-down

An altered stress response may be involved in the disruption and impairment of the brain and gut axis[41]. Research convincingly shows that unregulated stress early in life is a serious risk factor for developing various adult syndromes[36,43]; almost 80% of exposed young adults do not meet the criteria for successful psychosocial functioning in adulthood[44]. Such stress conditions occur when children are exposed to severe situations such as physical, psychological, or sexual abuse or family violence[45]. The consequences of an unmanageable event, such as a threat, are especially serious in a person with an early vulnerability or with a cumulative impact of negative life events. Such persons can have an abnormal sensitivity to any stimulus[45,46]. The support system in the hippocampus is constantly prepared for new traumatic experiences, as a sort of built-in "smoke detector"[46]. All of this can result in a long-term arousal of the nervous system, a hypersensitivity to stress and various psychosomatic/pathologic conditions in adolescence and adulthood[46].

Additional factors of the pathophysiology of IBS may include significant stressful life events as an adult or a stress reaction (fight or flight) that is repeated over time, become conditioned, and will start automatically in stressful situations[47,48], so-called conditioned stress.

Thus, an early vulnerability together with uncomfortable experiences can result in complex conditions in which emotional dysregulation, relationship problems, somatic stress and dissociation are encountered[49]. When neither fight nor flight reactions (the first more natural survival behaviours after a threat or stressor) are possible, the so-called freeze reflex (dissociation) may occur. Dissociation is the most severe condition related to dysregulation of the ANS[50]. It is important to understand the somatic manifestations of the stress response dissociation, as there is a close connection between psychological and somatic dissociation (when a patient cannot understand the messages from his or her body)[51,52]. Such emotional dysregulation may remain associated with certain regions of the body and never reach the conscious mind. Some

IBS patients request repeated somatic investigations and minimize the role of psychosocial factors[38]. This can be explained by their unconscious and somatic distress. Somatic dissociation results in decreased self-trust over time. This is a serious handicap in, which a patient's resources are completely exhausted; this can lead to burn-out syndrome without the patient's awareness[47,48].

Various emotions play key roles in altering autonomic and endocrine function, which in turn may derange the emotional circuitry[41]. The invalidation of emotional experiences as well as difficulty expressing and recognizing emotions (alexithymia) are often observed in IBS patients[49]. Gut function and pain sensation are centrally regulated by the emotions and the degree of awareness of the body's symptoms[40]. Thus the degree of the patient's body awareness is crucial. Long-term stress with distress, altered muscle tensions and repression of impulses can affect the ability to pay attention to the body and thus act on a person's body awareness[53]. Impaired body awareness has proven to be one cause of stress-related ill-health[54,55].

Bottom-up

Gastrointestinal microbial composition can be altered by infections, inflammations, diet or abdominal surgery[9,41]. All of these can affect different systems in the intestine. Several pathophysiological mechanisms, including visceral hypersensitivity, GI motility dys-function, intestinal inflammation, altered bowel microbial flora, and imbalance in the secretion of 5-hydroxytryptamine (5HT) have previously been reported[5].

Complexity

ANS dysregulation may, as mentioned above, occur by at least two different ways with alterations in many physiological reactions. A very complex interaction between factors may exist, and therefore trigger(s) of IBS syndrome can vary between patients. An imbalance in the ANS is seen in IBS but also in fibromyalgia, chronic fatigue syndrome and in interstitial cystitis. This raises the possibility that ANS dysfunction and/or chronic stress may be the common underlying pathogenesis[56-59]. Chronic on-going life stress can predict the development of IBS[36,58], and patients may experience symptoms up to 5-13 years prior to diagnosis[60]. Consequently, re-establishing balance in the ANS should be one important approach in the treatment of IBS[38].

ASSESSMENT BEFORE TREATMENT

Many authors emphasize the importance of a thorough examination of IBS patients with their many symptoms after excluding important somatic diseases[18,22,29,47,48,61]. In our studies, IBS patients are assessed *via* two physical examinations (one standing still and one during movement), blood and saliva samples after completing a thorough medical history. The patients complete questionnaires regarding gastrointestinal symptoms, psychological and psychosocial symptoms, and pain, dissociation, and quality of life and body symptoms[18,22,23]. In our study we found that gastrointestinal symptoms, body-oriented examinations and the patient's pain-drawings showed mostly deviating patterns, whereas the psychological and biochemical data were deviated or within normal limits during assessment before treatment[61]. In our experience to date, none of our more than 300 patients expressed only gastrointestinal symptoms.

Non-pharmacological treatments

IBS is a complex syndrome and most research concludes that the management of IBS should rely on a combination of non-pharmacological and pharmacological therapies as well as dietary and lifestyle modifications. Some authors claim that treatments involving interactions between body and mind are the most effective and thus the most powerful treatment strategies in IBS/body distress patients[37,62,63].

Various non-pharmacological treatment regimens have been used for IBS, including relaxation training, behavioural and psychological therapies, stress management, and meditation. Furthermore, body-mind therapies such as gut-directed hypnotherapy, mindfulness therapy, body awareness therapy and functional relaxation have been used with promising results both during treatment and at follow-up[18,22,29,64-83]. Table 1 summarizes various methods that have been used from the early 1980s until now. Over the years, these treatments have progressed from mostly individual to mainly group sessions; currently, there is a trend toward prolonged treatment sessions. The treatment modalities have also gone from focusing either on the body or the mind to now focusing on both. Cognitive therapies currently include body relaxation methods together with appropriate theories. Gut hypnotherapy adds body relaxation to mental exercises (guided imagination). Both therapies have reported responders and non-responders[81,82]. Mindfulness therapy and body awareness therapy consist of body movements inspired by Eastern philosophies, with the purpose of helping the patient to be present within the moment[83,84]. Physical activity, performed as supervised graded exercise training, has also had a positive effect on some patients with body distress syndromes such as fibromyalgia[36]. The ANS is reported to be influenced by breathing exercises and also by the use of movements such as Qigong, Tai Chi and yoga[48,60].

Gut directed hypnotherapy: Relies on inducing

Table 1 Overview of different non-pharmacological treatments for IBS patients

Ref/year	Treatment modalities
Stress management	
[66]/1987	PMR, thermal biofeedback, education, training in stress coping strategies, home practice, individual treatment
[67]/1991	Relaxation exercises (PMR), stress theory, individual treatment
Relaxation	
[68]/1993	PMR, home practice, individual treatment
[77]/2007	PMR, home practice (audio tape), small group treatment
Meditation	
[71]/2001	Relaxation response meditation, homework, individual treatment
Cognitive behaviour therapy	
[69]/2000	Cognitive education, PMR, isometric relaxation, home practice (audio tape), individual treatment
[70]/2000	Cognitive education, PMR, training assertiveness and coping strategies, individual treatment
[73]/2003	Biopsychosocial IBS theory, stress theory, homework, group treatment
[75]/2006	Psychoeducational theory, IBS theory, stress coping, homework diary, group treatment
Functional relaxation	
[79]/2010	For explanation se body text, small group
Mindfulness	
[80]/2011	Mindfulness stress reduction program specialized to IBS, *i.e.*, mindfulness training + cognitive behaviour theory, group treatment
[82]/2013	Mindfulness stress reduction with cognitive therapy program better than unspecified mindfulness alone, group treatment
Hypnotherapy	
[64]/1984, [65]/1987, [74]/2005	Hypnosis and PMR, audiotape daily, individual treatment
[72]/2002	Hypnosis and PMR, audiotape daily, individual treatment
[76]/2006	Guided imagery, PMR, individual treatment
[81]/2012	Hypnosis and PMR, audiotape daily, individual treatment
Body awareness therapy	
[18]/2002	Body awareness training, psychosomatic theory, IBS theory, group treatment
[22]/2007	Body awareness training, psychosomatic theory, IBS theory, group treatment

PMR: Progressive muscle relaxation; IBS: Irritable bowel syndrome.

a state of relaxation or trance (altered attention in the subject) in response to verbal or other stimuli, with suggestions for improvement made based on whatever condition is being treated[85]. The patient is taught relaxation, ego strengthening and coping skills. Tailoring the therapy to the patient's symptomatology is very important. The importance of practice cannot be over-emphasized and should ideally take place on a daily basis. It is often necessary to provide 12 sessions of treatment to gain maximum benefit. According to the author, this is a technique that is exceptionally operator-dependent[85].

The key aspects of mindfulness are to observe without reacting to internal sensations and to pay emotionally neutral attention to all experiences, impressions, thoughts and feelings[80]. It is also important to be fully present in all activities and have a non-judgmental approach to life experiences. Adaption of this practice to an IBS population was done by emphasizing the relevance of mindfulness in coping with IBS-related symptoms and perceptions. Participants are instructed to notice any sensations in the abdominal area and to distinguish those sensations from thoughts about the sensations. Instruction and homework assignments are related to body scan, sitting and walking meditation, and mindful yoga[80].

Body awareness therapy: Body awareness therapy (BAT™) consists of simple structured movement exercises based on human anatomical and physiological prerequisites to achieve optimal movement dynamics[54,55,84]. The BAT™ exercises aim to help the body find its natural posture, thus facilitating the circulatory, muscular, nervous and breathing systems to recover their natural function. By doing so, unconscious physical and psychological experiences will be brought into awareness and can be dealt with both physically, mentally and verbally. BAT™ was developed by Swedish physiotherapists in the early seventies, and it is now used for treatment of various pain and stress-related conditions in all Nordic countries, as well as in Scotland, Switzerland, Austria, the Netherlands, Spain and Turkey[84].

The assumed mechanism of functional relaxation is the treatment of somatoform autonomic dysfunction with proprioception[79]. Very subtle movements of small joints are performed during relaxed expiration, which is accompanied by focusing on and exploring the perceived differences in body sensations triggered by these movements. This takes unconscious physical/psychological experiences into account and, as basic motivational systems are rediscovered and further developed, early forms of bodily self-awareness can be re-experienced.

One common goal of these four methods is to learn how to be aware in the present, to be in the here and now. The posture, breathing, and level of muscular tension together with the function and mobility of the inner organs are affected by body-mind training. Bodily experiences always exist within the present, awareness of emotions is inseparable from the consciousness of their bodily expressions and together, all of these express how a person feels physically and mentally. In this way, body-mind therapies are assumed to work through a physiological transformation accomplished *via* the autonomic nervous system[53,84]. Although the methods differ slightly in how they are addressed, either through the mind (hypnotherapy and mindfulness) or through the body (body awareness therapy

and functional relaxation), the treatment results are similar.

Aspects of performing body-mind therapies

Hypnotherapy has been used in IBS patients with good results since Whorwell *et al*[64] introduced it in 1984. Hypnotherapy has been used mostly with gut-directed therapy and mostly on an individual basis; patients are given an audio-tape for homework. However, according to Whorwell *et al*[85], 2006, it is a labour-intensive modality with a finite success rate and is not suitable for everyone. He suggests that it is best incorporated into a program of graduated care that has a contingency plan for dealing with individuals who do not respond to this particular form of treatment[74]. Improvement in symptoms with hypnotherapy is largely sustained, although some patients may require occasional "top-up" sessions to maintain their improvement[74]. Carolusson and her team also used individually tailored techniques but included both gut-oriented hypnotherapy and hypnoanalysis either separately or in combination[29]. The author conclude that the hypnosis treatment has to be designed depending on the patients' personality and possible mental defence-functions in relation to the symptoms as well as the patients' mental and social resources[29].

When treating patients with body awareness therapy in a group, one has to emphasize that each person concentrate on and listen to her own body and not to carry out any movement that does not apply to her. In allowing the body, and not the mind, to do what it wants, one can find a way out of pain[54,55]. The ability to listen to the body might be severely impaired in patients at the beginning of treatment. By suggesting that patients try different ways of performing an exercise, the therapist(s) can help the patients find what is comfortable for them[18,22,55]. A good working alliance and safety in the treatment situation are important for change to take place[47,48,61,86].

When treating IBS patients with a tendency to dissociate, the therapist must be careful not to re-victimise the patient and thus risk the patient dropping out[86]. By noticing early warning signs for dissociation and with careful guidance, the patients will learn how to build a trusting relationship with themselves and others, to maintain a psychological as well as a physical integrity (maintaining boundaries) and to gradually find words to describe the body's signals and sensations. Thus, with increasing body awareness, the patients learn how to stabilize themselves when emotional systems are aroused[47,48,61]. To first perceive the body and then to connect the sensations in the body with a certain sense or emotion is crucial for the treatment to be effective[22,61,84,87]. The patient may express after several treatments: "Before I just had a stomach ache, but now it is like that just before I get pain, I feel angry".

Duration of body-mind treatment

The length of treatment can be crucial[18,22,29,61]. A short treatment duration is not always sufficient for all patients; some can be left behind as they display more symptoms[18,22,29,61]. In our studies, we have found that there can be different patient treatment processes[61]. IBS patients grade themselves on different symptom questionnaires, and body and biochemical parameters are assessed; the process can be determined by these parameters. For example, one patient estimated high levels of symptoms before treatment that were reduced after treatment. Another patient who started out by estimating low levels before treatment scored higher at 12 wk and then lower again at 24 wk. This patient probably needed more time to become aware of bodily sensations, and thus "underestimated" the levels before the treatment start. A third patient may score increasing symptoms during the entire treatment period. This is an example of a patient who started out with a very low body awareness, whose experiences have been out of reach in the body and slowly emerged to awareness during treatment. Hence, treatment of this patient should not be concluded until the symptoms decrease[22,29].

In another study, some patients' symptoms worsened after 12 wk with 1-h treatments each week[29]. Some authors suggest that a treatment period of 12 wk was not long enough to achieve deep, long lasting improvements[29,37]. Treatment can potentially uncover denied or dissociated suffering, leading to a period that can be painful, sad, and heavy for the patient[29]. In our studies (24 wk with 2 h weekly) we have found that these periods mostly occur between 8 and 12 wk of treatment for most of the patients[18,22,61]. In hypnotherapy, Gonsalkorale *et al*[72] showed that males with D-IBS showed lower results with hypnotherapy at 12 wk of treatment than did females. We found that 12 wk of treatment with body awareness therapy was not enough for D-IBS patients, who needed 24 wk, and that these patients showed lower body awareness at their first assessment[18,22]. Our theory was that they needed 12 wk to increase their body awareness and then the rest of the treatment time to restore it[18,22]. One study noted that although the treatment reduced distress such as anxiety and depression, it did not affect gastrointestinal symptoms[70]. In our experience, reduction of the distress occurs prior to the reduction of gastrointestinal symptoms[61].

Working relationship between patient and therapist

A number of authors emphasize the importance of a good working treatment relationship between the patient and the clinician/therapist[38,70,74,88,89]. The therapists need to learn how to create practicable channels of contact[48]. A person with a cognitive orientation wants to obtain a theoretically plausible explanation of her problems in order to feel secure.

A person with alexithymia traits, who does not have words for emotions and cannot express them, needs to be confident with her body and increase her body awareness. A person with a vivid and colourful imagination is probably susceptible to exercises that include mental visualization. An optimal treatment plan should comprise all of these components[47,48]. A treatment structure with body awareness movements integrated with theory and reflections may be one appropriate treatment design[18,22,61]. For example, to a traumatized person, a description of trauma and its effects on the body can provide an explanation for the inexplicable symptoms she experiences. A patient may say, "When I come home to mom's street, I felt that something bad had happened here, but I do not know what it is". Another patient may allow herself to remember the terrible things that happened during her early years and finally put it into words. Histories of abuse are not always volunteered by patients[38].

Results of body-mind treatment

Many studies have shown that the patients' gastrointestinal symptoms and the extra-intestinal manifestations improve along with increased body awareness. This is in sharp contrast to pharmacological approaches, in which often relatively few symptoms resolve[72]. Patients develop better relationships with their own body and with life around them. As exemplified by one patient; "I can now notice how I am sitting, standing and walking, if I am anxious or relaxed and also that feelings quickly transmit to the stomach". The patients change from feeling controlled by their gut and their symptoms to feeling safer and able to handle different situations in life, both physically and mentally. They may say, "I now recognize when the stress in the body speeds up and can stop in time". Patients with gastroenterological problems can affect their symptoms with suitable exercises: "I have great use of the exercises that I learned at our meetings; I practice them daily and have now only minor phases of pain from my diverticulitis"[61].

In our studies, the leaders noted recurring indications during treatment that patients became more relaxed and more grounded. We observed, for example, decreased facial tension and better balance in movement. The patients developed a better relationship with their own bodies, which, among other things, was noticeable when they found it easier to relate to their own body and expressed more positive opinions about it. The patients also scored lower levels of psychological symptoms such as depression, obsession, anxiety, anger and phobias after treatment. As the patients became more aware of their symptoms, they could improve their body awareness and their symptoms decreased. In the group situation, the changes were also reflected. For example, patients who were silent earlier became more confident and started talking more in the group, and those who had not previously taken part in pair exercises now participated. They also showed more assertiveness and self-esteem, expressed as: "I have noticed, that I am more clear with how I would like to have it, I stand up for myself, and I get a greater response". Some patients reported that they had stopped dwelling on injustices from the past and could release these and move on[61].

DISCUSSION

Many authors, including Collins *et al*[30] in 1948, Bargen *et al*[31] in 1956 and Enck *et al*[33] in 2008, stressed that IBS is a complicated condition with both physiologic and psychological factors involved in the pathogenesis. According to Gonsalkorale *et al*[72] in 2002, IBS has gained the reputation of being somewhat unrewarding to treat. As a consequence, physicians are inclined to adopt the approach of ensuring that there is nothing "seriously wrong" by a process of thorough investigation but not necessarily offering help in terms of how to cope with the condition. Many of the patients, especially the severe cases, have lost their confidence and feel like "failures" with no hope when they come to an assessment[61,72]. Because IBS patients also exhibit a variety of symptoms, they find it difficult to "fit in" within the normal health care system with its high degree of specialization. For example, within the field of gastroenterology, hospitals may have different departments for the upper and lower gastrointestinal tract. This involves a great risk that patients with multiple symptoms and multiple diagnoses may fall in between categories, and that their treatment will be inadequate. However, by adopting a graduated treatment program with a team approach to management, extremely high levels of satisfaction in patients and fulfilment in staff can be achieved[72].

The key to effective treatment strategies for these multi-symptomatic IBS patients is to understand the heterogeneity of the disorder. A pathophysiological explanation may be that ANS dysregulation occurs due to conditioned chronic stress or emotional stress (traumas) experienced early in life or later on. The emergence of ANS dysregulation may also be caused by a straightforward effect on the gut. Thus, a question may arise as to whether we can give these different types of IBS patients the same non-pharmacological treatment, or if we should differentiate the treatment for different types of IBS. One study has shown that approximately 25 percent of patients repressed somatised psychological problems and needed insight oriented hypnotherapy in addition to gut-directed treatment[29]. Similar proportions have been reported by others[88,89].

A comprehensive body examination can give us a hint about the non-pharmacological treatment duration needed for a patient to improve. When IBS patients are either treated too briefly or with a treatment that

is not optimal, the patient may experience relief from some symptoms, but the underlying distress present in quite a few of these patients will remain untreated and can be replaced by other symptoms (known as symptom shift)[29]. The risk is that their underlying problems will be expressed in new ways, and that the patients will therefore seek treatment elsewhere without ever understanding their internal body-mind communication[29]. It is the opinion of many authors that treatment should be carefully chosen after a thorough examination of each patient and that treatment should target all of the symptoms[21,72,85].

Those patients who need longer treatment durations may be patients defined as non-responders, males with D-IBS or those who have severe social stress; these factors are likely to detract from the efficacy of the treatment[85]. One suggestion for the lack of improvement in males with diarrhoea was that they had somewhat lower hypnotic or imaginative abilities compared to females[85]. Another possible cause for the lack of improvement could be that these males with D-IBS had lower body awareness from the beginning. We know that D-IBS patients with lower body awareness have prolonged recovery times[18,22]. However, the results in that study did not separate men and women because of the low number of men enrolled.

Some patients will experience relief from some symptoms but not always the gastrointestinal symptoms first. Hypnotherapy, body awareness therapy and mindfulness treatment will almost certainly improve their coping skills in life situations. It is unclear whether these body-mind therapies such as hypnotherapy (guided imagery), mindfulness and body awareness therapy have something in common or are separate entities[89]. These methods involve the body by relaxing the muscles or by normalizing muscle tension, and they also emphasize the importance of being present in the moment. Only in the present one can access and influence the experience and behaviour patterns of the body/mind, which are established in the nervous system[90]. A plausible consequence of this is that consciousness of the "here and now" is essential for changing processes and should be the focus of therapy from the beginning[90]. The patients must first become aware of the present moment and the elements of their experiences of both the body and the emotional sphere in its entirety. Then, they understand that they need to learn to react differently to alarming situations that otherwise can be made worse by their response to it. This educational process is one part of the therapeutic package together with body awareness. The technique can thus be used to control symptoms and to reduce psychological distress and improve coping skills[61,90].

There is a general consensus that the health problems that will dominate in the future are psychosomatic or psychosocial disorders or diseases[91]. It has also been suggested that in the public medical service one cannot make use of the same diagnosis and treatment that is used for welfare diseases. This should cause us great concern, and we need a new approach for these patients. Good teamwork is important during this new approach to treat multi-symptom patients. Therapists should be encouraged to discuss IBS cases with each other and also with the physicians included in the team to ensure that any real or potential medical problem that may arise can be promptly resolved[61,85].

CONCLUSION

The pathophysiology of IBS syndrome likely depends on autonomic dysfunctions that can affect the patient both "top-down" (from the brain to the gut) and "bottom-up" (from the gut to the brain), leading to multiple symptoms such as increased intestinal sensitivity and motility dysfunction. In addition, psychological distress enhances these symptoms. The key to planning effective management strategies is to understand the heterogeneity of this disorder. Thus, treatment should be focused on a body-mind intervention directed by a good assessment survey of the individual patient both by a gastroenterologist and a body-mind therapist. The duration of the treatment should be adjusted according the needs of the individual patient.

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REVIEW

Dysbiotic infection in the stomach

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Abstract

Microbiota in human alimentary tract plays important roles for homeostatic maintenance of the body. Compositional difference of gut microbiota is tightly associated with susceptibility of many diseases, including inflammatory diseases, obesity, diabetes mellitus, cancer, and atherosclerosis. "Dysbiosis" refers to a state of imbalance among the colonies of microorganisms within the body, which brings abnormal increase of specific minor components and decrease in the normally dominant species. Since stomach secrets strong acid for its digestive role, this organ has long been thought a sterile organ. However, the discovery of *Helicobacter pylori* (*H. pylori*) has changed the concept. This bacterium has proven to cause gastritis, peptic ulcer, and gastric cancer. However, recent cross-sectional studies revealed that *H. pylori* carriers had a decreased risk of developing immunological diseases, such as asthma. *H. pylori* coinfection also suppresses inflammatory bowel diseases. This review describes human gastric microbiota by discussing its mutual interaction and pathogenic enrollment. Gastric "dysbiosis" may affect host inflammatory response and play important role for gastric pathogenesis. We will topically discuss enrollment of dysbiosis for genesis of gastric cancer as well as for disruption of immunological homeostasis affecting oncogenic resistance.

Key words: Stomach; Microbiota; Dysbiosis; *Helicobacter pylori*; Epstein-Barr virus

Core tip: The imbalance of microflora in the gut induces dysbiosis. Altered gut microflora is known to be associated with inflammatory diseases, obesity, diabetes, cancer, and atherosclerosis. Little is known about gastric microflora, which will also interacts with bacteria, viruses and funguses. In this review, we discuss that dysbiosis in the stomach may disrupt

immunological homeostasis, reduce of carcinogenic resistance, and induce gastric cancer.

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INTRODUCTION

Various microbes, from commensal to pathogenic, reside in the human body. Not only they are interacting with their host, but also these different microorganisms (bacteria, yeast, viruses, parasites, *etc*.) are interacting with each other. This interaction sometimes causes dysbiosis, which refers to microbial imbalance inside the body. Dysbiosis in the digestive tract sometimes exacerbate bowel disease[1].

Microbial colonies found in human body are normally beneficial, but are parasitic, commensal, or symbiotic. These appropriately sized microbial colonies assist necessary functions in digestion. The beneficial bacterial colonies also protect the body from the penetration of pathogenic microbes by competing with pathogens for space and nutrition. Dysbiosis in bacteria refers to increased levels of harmful bacteria and reduced levels of the beneficial bacteria.

The microbial interaction also occurs between bacterium and other microbes, such as virus and fungus. Bacteria and viruses residing together can work sometimes synergistically to enhance pathogenesis. Gene expression of viruses showing latent infection, such as Kaposi's sarcoma-associated herpesvirus, Epstein-Barr virus (EBV), and HIV, is influenced by epigenetic modifications induced by bacteria[2]. Latent infection of these viruses can be disrupted by bacterial products and viral production will be reactivated (Figure 1). In HIV-positive persons, immunosuppression promotes growth of other opportunistic organisms that contributes progression of acquired immune deficiency syndrome (AIDS). In addition, bacterium-virus interactions should be involved in oncogenic process. Both *Helicobacter pylori* (*H. pylori*) and EBV are associated with gastric cancer, respectively[3,4]. Since *H. pylori* spreads in many human populations and its roles for stomach cancer development is well accepted. Infection of EBV into gastric epitherial cells also develops gastric cancer in fewer than 10% of total cases, which often associates with lymphoplasmacytic infiltrate.

This paper will be focused on dysbiotic infection in the stomach. Although not as many as lower alimentary tract, some microbes reside in the stomach. Some are derived from dietary intake, others are from oral, nasopharyngeal, and tracheal swallows. Duodenal reflux will also bring microbes to the stomach. Dysbiosis in the stomach will bring imbalance to immunological homeostasis, which may take some part in inflammatory response and will be involved with pathogenesis. The effects of coexisting bacterial-bacterial and bacterial-viral coinfections should be considered for pathogenesis of gastric diseases.

GASTRIC ACIDITY, *H. PYLORI*, AND OTHER BACTERIA

The gastric juice represents a barrier to microbes in saliva and ingested food, mostly due to the degenerative activity of hydrochloric acid[5]. If this bactericidal activity is weakened by an elevation of gastric pH, microbes will be allowed to survive in the stomach. It is reported that 80% of healthy subjects between 80 to 91 years old showed hypochlorhydria with pH 6.6. These people posessed 10^5 to 10^8 colony forming units per ml of bacteria in fasting gastric aspirate[6]. The strong association between diminished gastric acid secretion and the presence of opportunistic enteric pathogens was clearly observed in AIDS patients[7]. The gastric barrier to infection has more significant meaning to hosts of whom immunological defense is weakened.

We showed when *H. pylori* was mixed with an acid resistant isolate of *Kingella denitrificans* (*K. denitrificans*), a commensal of the human respiratory tract, survival of *H. pylori* in acidic condition was increased compared with the single culture of *H. pylori*[8]. Binding of the acid resistant *K. denitrificans* with *H. pylori* seemingly coated the bacterial body to allow survival of *H. pylori* in the acidic condition. Another studies have revealed that commensal and foreign microbes may interact intimately with gut epithelium to influence host signaling pathways that regulate metabolic and stress responses[9,10]. The colonization of commensal microbes in gastric epithelium may affect the carcinogenic potentials of *H. pylori* by modulating CagA-mediated regulation of oncogenic signals.

GASTRIC MICROBIOTA

Thick mucus layer, acidic gastric juice and peristaltic movement in the stomach have raised the dogma that "the stomach is a sterile organ". However, the dogma quickly changes after the discovery of *Campylobacter pyloridis* in 1982, which is renamed into *H. pylori* in 1984[11]. *H. pylori* can colonise the stomach by producing urease to survive under acidic condition. Soon after the discovery of *H. pylori*, another type of bacteria such as *Vellomella*, *Lactobacillus* and *Clostridium* are found as transient bacteria that reside in the stomach[12]. However, the ability of the transient bacteria to crosslink with the host and penetrate the mucosa layer is drawing people's attention.

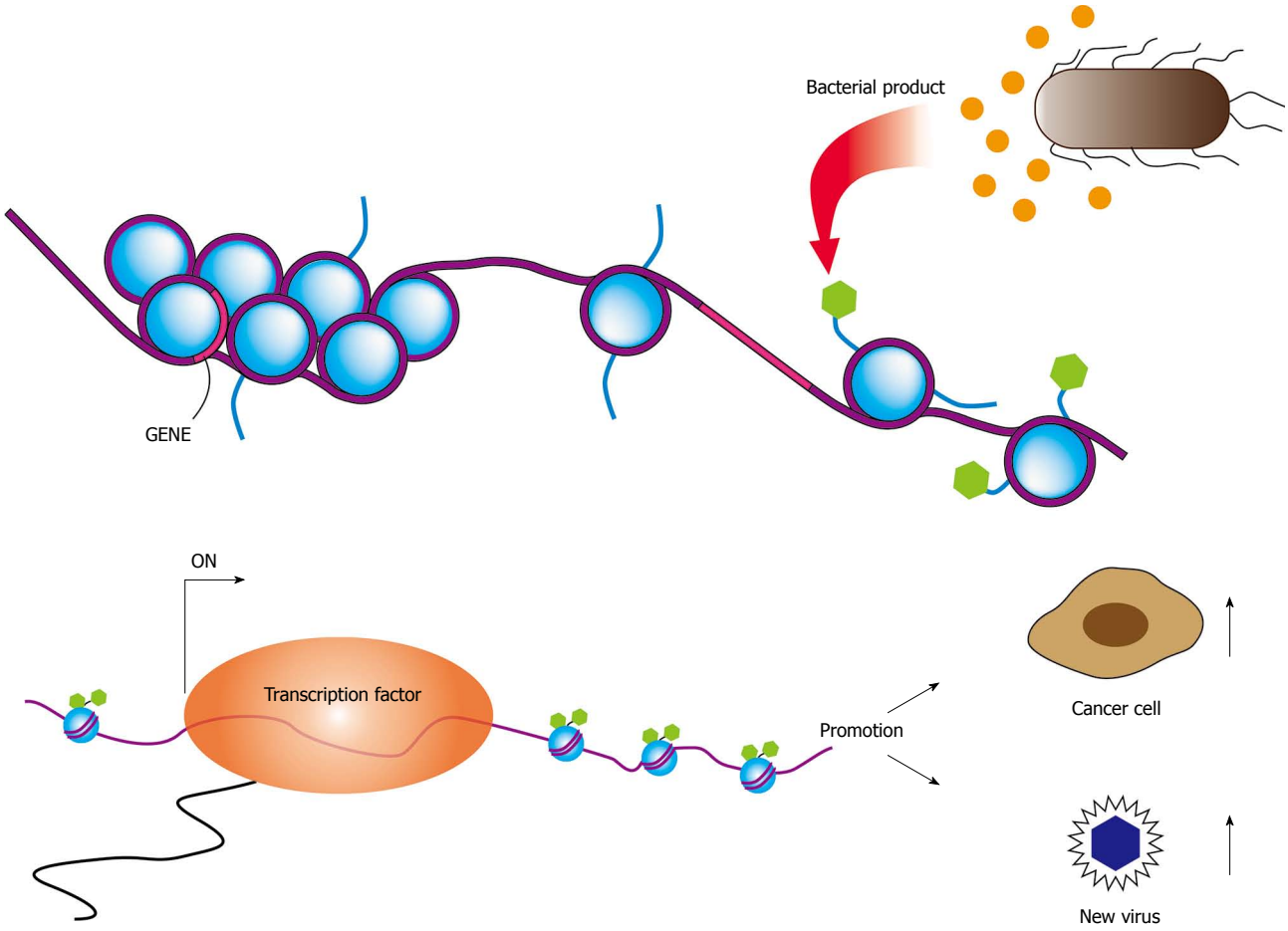

Figure 1 Epigenetic modifications to promoters. Epigenetic modifications to viral or cellular promoters regulate expression of human and viral genes. Bacterial products and or proinflammatory cytokines activate epigenetic marks on viral or cellular promoters, which can promote viral production as well as stimulate the transcription of viral oncogenes. These epigenetic modifiers also stimulate cellular proliferation. The reactivation of a latent virus results not only production of virion, but also may drive cellular transformation.

Recently, the development of culture-independent molecular technologies based on 16s rRNA has revealed that five abundant genera microbiota other than *H. pylori* reside in the stomach. They are *Neisseria, Haemophilus, Prevotella, Streptococcus,* and *Porphyromonas*[13-16].

Dysbiosis of the gastric microbiota has been implicated in immune system regulation and enhancing disease symptoms. Several researchers showed the gastric microbiota arose from patients infected with *H. pylori* are different from uninfected people[17,18]. Osaki *et al*[19] also described the prolonged exposure to *H. pylori* infection has altered the composition of the microbiota in rodent stomachs. These findings suggested an interaction between *H. pylori* and the gastric microbial community[8]. Though the mechanism of *H. pylori* in altering the gastric microbiota remains unclear, possible explanation is that the induction of host antimicrobial peptides, such as β-defensin 2[20] or cecropin-like peptide, may directly kill another microbiota[21].

All of these findings had shed a light that dysbiosis of gastric microbiota might related to the susceptibility to gastric inflammation and tumorigenicity in patients with *H. pylori* infection. *H. pylori* infection also initiate the inflammatory cascades that induce physiological changes that reduces the gastric secretion from parietal cells and elevation of pH in the stomach. The elevation of pH eventually resulted in the colonisation of another microrganisms in the stomach[22-25]. Engstrand *et al*[26] reported that gastric cancer development may related to the alteration of gastric microbiota. The commensal microbes can communicate with dysbiotic pathogens such as *Salmonella typhimurium* that have the ability to alter gastrointestinal homeostasis to pathogenic inflammation. However, it should be further investigated whether infections with commensals are associated with the susceptibility to gastric inflammation and tumorigenicity in patients with *H. pylori* infection.

INFECTION AND GASTRIC CANCER

H. pylori is a primary causative agent not only for peptic ulcer diseases and chronic gastritis, but also for gastric cancer. Other than *H. pylori,* EBV is also known to cause gastric cancer. EBV-associated gastric carcinoma (EBVaGC) comprises about 10% of all

gastric carcinomas worldwide[27,28]. *H. pylori* infection has been linked to CpG hypermethylation of tumor suppressor genes, including Runx3, E-cadherin, and p16[29-32]. EBV infection was correlated with overexpression of DNA methyltransferase 1 in gastric cancers[33]. And EBVaGCs have a unique pattern of methylation linked to the downregulation of p16 but not MLH1[34,35]. High methylation frequencies of several tumor suppressor genes, APC, PTEN, and RASSF1A, and cell adhesion molecules, THBS1 and E-cadherin, were also reported in EBVaGC. The posttranscriptional modification might change the epithelial phenotype, important for generating gastric microbial niche, however, it is too early to discuss effect of such alteration for gastric microbiota. On the other hand, several reports describe synergy between *H. pylori* and EBV for the genesis of gastric cancer. Firstly, individuals co-infected with *H. pylori* and EBV significantly possessed severe inflammatory lesions than persons with a single *H. pylori* infection[36]. It has also been shown that *H. pylori* infection was associated with EBV reactivation in patients with gastric symptoms[37]. Lastly, reactivation of EBV in latently infected gastric epithelial cells was induced by monochloramine, a product of *H.pylori* infection[38]. These observations suggest that coinfection of the two pathogens possibly heighten risk of gastric cancer[39,40].

H. pylori-related gastritis frequently initiates in the antrum. On the other hand, EBVaGC tumors are frequently located near the mucosal atrophic border, where mild to moderate atrophy is common[41]. Both EBV and *H. pylori* could be abundantly detected in the same mucosa of patients suffering with moderate chronic atrophic gastritis, where inflammatory cell infiltration is abundant. However, neither microbe could be frequently detected in the mucosa with marked atrophic gastritis, where inflammatory cell infiltration is scarce[34]. The observation strongly suggested inflammation caused by bacterial infection might promote generation of cancer associated with EBV infection[4,35].

Gastric remnant cancer arises after distal gastrectomy for benign disease, which includes refractory gastric or duodenal ulcer disease and recurrent ulcer with gastric outlet obstruction. The incidence of gastric remnant cancer ranges from 1% to 7% of all gastric carcinomas[42]. Gastric remnant carcinoma is characteristically associated with EBV infection in high frequency (25% to 41.2%). It is considered that the reflux of bile and pancreatic juice causes regenerative atypia and cell proliferation in epithelial cells[43]. In Billroth-II anastomoses, atrophic change of remnant gastritis is frequently accompanied by EBV-positive gastric remnant carcinoma[34,44].

EBV efficiently drives proliferation of human primary B cells *in vitro*, which subsequently transforms B cells. B-cell proliferation is also driven by ligands of Toll-like receptors (TLRs). Proliferation of EBV-infected B cells and their capability to interact with immune effector cells may be directly influenced by components of bacteria or other microbes present at the site of infection[45,46]. Oral commensal *Porphyromonas gingivalis* produces butyric acid, which may reactivate epigenetic silencing by increasing H3 and H4 acetylation[47]. It is well known that EBV transactivator, BZLF1 same as ZEBRA or Zta, which reactivates latent infection of EBV to lytic replicative infection, can be induced by treatment of latently EBV-infected cells with butyric acid[48]. These reports suggest dysbiotic bacterial infection activates latently infected viruses, which exacerbate microbial infection (Figure 2). These observations strongly remind us of the idea that cancer associated with inflammation.

POST-INFECTIOUS IMMUNE-DISORDER IN UPPER GASTROINTESTINAL TRACT

Infection and immune dysregulation in intestinal tract

Exposure to acute gastrointestinal infection induces persistent low grade mucosal inflammation, which sometimes leads to onset of post-infectious irritable bowel syndrome (PI-IBS)[49-53]. Organisms such as *Campylobacter*, *Salmonella*, *Escherichia coli* (*E. coli*), and *Shigella* are common pathogens involved in the development of PI-IBS. Immune disorders found in PI-IBS patients are characterized by mucosal infiltration of immune cells, including macrophages, T cells, mast cells, and eosinophils, as well as increased production of various cytokines[49,50,52,54-58]. TLR-dependent innate immunity is also activated along with persistent low grade gut inflammation following acute gastroenteritis (AGE), which may be associated with dysbiosis of gut microbiota[59-62].

Functional disorders following AGE in upper gastrointestinal tract

Functional dyspepsia (FD), a main functional disorder in the upper gastrointestinal tract, can also develop in previously asymptomatic individuals following an episode of microbial infection-related AGE. This type of FD is currently recognized as post-infectious FD (PI-FD)[63,64]. Tack *et al*[65] reported that 55 (17%) cases from 400 FD patients had episodes of AGE, while PI-FD onset was not correlated with the rate of *H. pylori* infection. A prospective observational study evaluated the incidence of FD development in patients with *Salmonella* infection-induced AGE after 1 year. The FD incidence was significantly higher in the infection cohort (13.4%) as compared to the non-infection cohort (2%)[66]. The systematic review including meta-analysis findings was performed at more than 6 months after AGE. The mean prevalence of FD following AGE was 9.55% in adult populations. The summary odds ratio for development of PI-FD was 2.54 (95%CI: 1.76-3.65)[67]. The pathogens *Salmonella spp.*, *E. coli O157*, *Campylobacter jejuni*, *Giardia lamblia*, and *Norovirus* have all been associated with

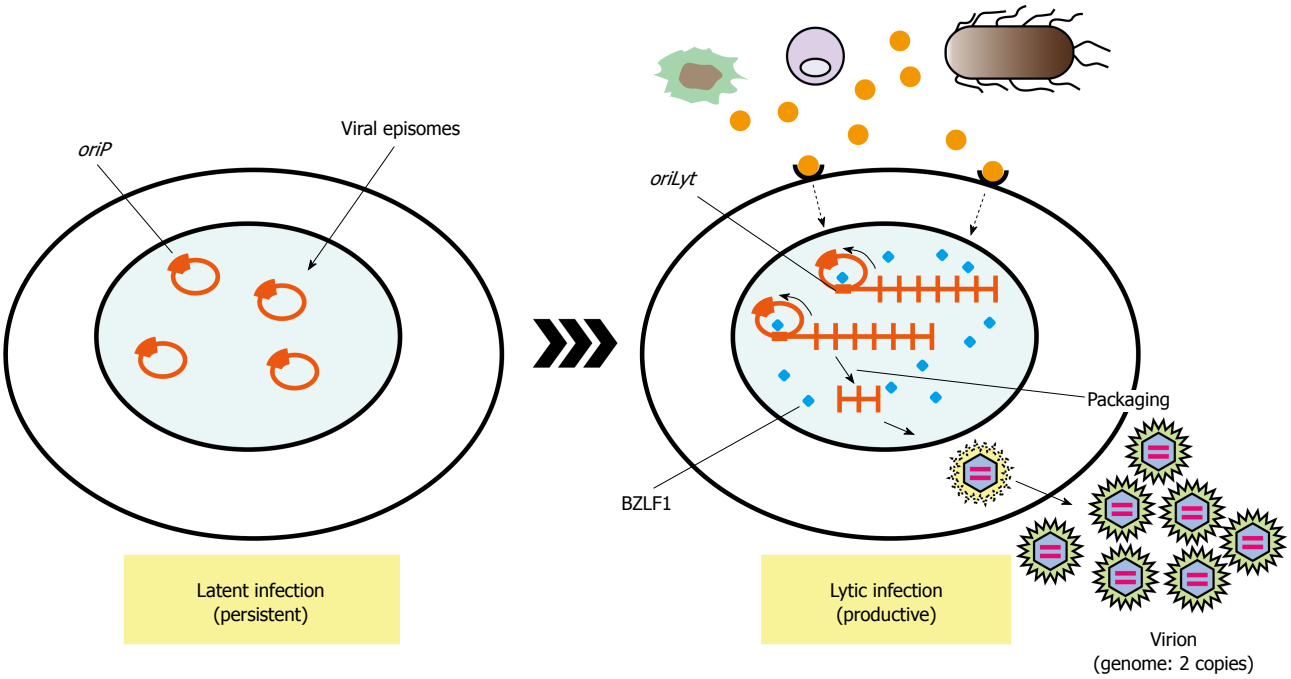

Figure 2 Lytic activation of Epstein-Barr virus by inflammatory product. After primary infection of EBV, the infected cells undergo prelatent cycles in which only immediate-early and early genes are expressed with no viral production. This transient lytic state is silenced, and latent infection is persistently established by expressing only limited numbers of latent genes. The latent infection may undergo the lytic cycle, in which viral late gene expression, viral genome replication, and production of the progeny virus (virion) can be observed. BZLF1 is a molecular switch for EBV reactivation from latent infection. And various signaling pathways activate cis-acting elements in the BZLF1 promoter[47,48]. Although viral latent gene such as LMP1 can also strongly expressed on lytic infection, which sometimes promote cell proliferation by enhancing cell signaling, modulating immune system, and inducing genomic instability. *oriP* is a latent origin for viral genome replication. BZLF1 is a transactivator of virus replication, which forms homodimers and binds to *oriLyt*, origin for EBV DNA replication in lytic infection. EBV: Epstein-Barr virus.

the development of PI-FD.

Altered populations of epithelial and mucosal immune cells in upper gastrointestinal tract following AGE

Although AGE may be one of the crucial causes in the development of PI-FD, its pathogenesis has not been fully investigated. AGE was shown to induce persistent low-grade mucosal inflammation via altered immune functions in the upper gastrointestinal tract (Table 1). Kindt *et al*[68] reported aggregation of $CD3^+$ T cells, decrease of $CD4^+$ T cells, and increase of $CD68^+$ macrophages along the muscularis mucosae of duodenum in PI-FD patients. Increased infiltration of CC chemokine receptor-2^+/$CD68^+$ macrophages and eosinophils in duodenal mucosa was also found in certain populations of FD patients[69]. The number of mast cells and enterochromaffin cells in gastric mucosa was significantly increased in PI-FD patients than FD patients with no episodes of AGE[70]. In addition, increased number of mast cells and enterochromaffin cells is often found in the colonic mucosa of PI-IBS patients. Apart from bacterial and viral infections, the incidence of PI-FD was increased in patients with a history of parasitic *Giardia* infection. Moreover, the number of cholecystokinin-producing enterochromaffin cells was increased, but the number of serotonin-producing enterochromaffin cells was decreased in the duodenal mucosa of giardiasis patients[71]. The pathogenesis of PI-FD may be influenced by altered populations of immune cells as well as serotonin metabolism in the upper gastrointestinal tract. However, the detailed mechanisms of PI-FD remain to be fully clarified.

Is post-infectious immune-disorder in the upper gastrointestinal tract associated with dysbiosis?

Dysbiosis of the gut microbiota has shown to be associated with the pathogenesis of intestinal inflammatory and functional disorders. AGE certainly plays an important role in the pathogenesis of PI-FD through an immune disorder in the upper gastrointestinal tract. However, it remains largely unknown whether AGE directly induces dysbiosis or only influences the process of development of PI-FD. Inflammasomes regulate gut microbiota by co-functioning with various inflammatory signals from cytokines, such as interleukin-1β and 18, as well as with signals from TLR-4 and TLR-9 innate immune receptors[72]. AGE-associated induction of dysbiosis may be regulated by such processes, however, further investigations are required to elucidate the role of infection-induced dysbiosis and its association with functional disorders in the upper gastrointestinal tract.

CONCLUSION

It has been proven recently that not only long-term dietary intake, but also short-term dietary intake alters

Table 1 Altered populations of epithelial and mucosal immune cells in post-infectious functional dyspepsia patients

Ref.	Location	Cell population	Changes
Kindt *et al*[68]	Duodenum	$CD3^+$ T cells	Aggregated
		$CD4^+$ T cells	Decreased
		$CD68^+$ cells (macrophages)	Increased
Futagami *et al*[69]	Duodenum	$CCR2^+/CD68^+$ cells (macrophages)	Increased
		Eosinophils	Increased
Li *et al*[70]	Stomach	Mast cells	Increased
		EC cells	Increased
Dizdar *et al*[71]	Duodenum	EC cells (5-HT-producing)	Decreased
		EC cells (CCK-producing)	Increased

EC: Enterochromaffin; CCR: CC chemokine receptor; 5-HT: Serotonin; CCK: Cholecystokinin.

human gut microbiome[73]. The animal-based diet significantly increased the levels of fecal deoxycholic concentrations, which is the product of microbial metabolism and promotes liver cancer[74]. Moreover, the animal-based diet significantly increased sulphite-reducing bacteria which might increase inflammation to intestinal tissue through H_2S production[75].

Human disease can also be developed from an imbalance between commensal bacteria and fungi[76]. *Candida albicans* (*C. albicans*) extensively distributes on human skin and mucosal surfaces, such as the oral cavity, the gastrointestinal tract, and the lower female reproductive tract. Because of this, the fungus is most frequently implicated in mixed bacterial-fungal infections. Enhancement of bacterial virulence by *C. albicans* has been described in studies assessing the virulence of mixed *C. albicans* and *Staphylococcus aureus* infection in mice[77].

Bacteria, virus, fungus, and some parasites are affecting each other to reside and propagate in human alimentary tract. Their opportunistic imbalance often provides illness to human beings. Our body had better keep benign microbiota and refrain from having dysbiotic microbiota. Though little in known, further investigation will surely tell us the way how to keep symbiotic relation with gastric microbiota.

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Intra-abdominal drainage following pancreatic resection: A systematic review

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Abstract

AIM: To study all the aspects of drain management in pancreatic surgery.

METHODS: We conducted a systematic review according to the PRISMA guidelines. We searched the Cochrane Central Registry of Controlled Trials, EMBASE, Web of Science, and PubMed (MEDLINE) for relevant articles on drain management in pancreatic surgery. The reference lists of relevant studies were screened to retrieve any further studies. We included all articles that reported clinical studies on human subjects with elective pancreatic resection and that compared various strategies of intra-abdominal drain management, such as drain *vs* no drain, selective drain use, early *vs* late drain extraction, and the use of different types of drains.

RESULTS: A total of 19 studies concerned with drain management in pancreatic surgery involving 4194 patients were selected for this systematic review. We included studies analyzing the outcomes of pancreatic resection with and without intra-abdominal drains, studies comparing early *vs* late drain removal and studies analyzing different types of drains. The majority of the studies reporting equal or superior results for pancreatic resection without drains were retrospective and observational with significant selection bias. One recent randomized trial reported higher postoperative morbidity and mortality with routine omission of intra-abdominal drains. With respect to the timing of drain removal, all of the included studies reported superior results with early drain removal. Regarding the various

types of drains, there is insufficient evidence to determine which type of drain is more suitable following pancreatic resection.

CONCLUSION: The prophylactic use of drains remains controversial. When drains are used, early removal is recommended. Further trials comparing types of drains are ongoing.

Key words: Pancreas; Pancreatic resection; Pancreatectomy; Drainage; Pancreatic fistula

Core tip: This systematic review updates our current knowledge on the management of intra-abdominal drains in pancreatic surgery. Regarding the prophylactic use of intra-abdominal drains, current studies do not lead to definite conclusions whether routine drainage should or should not be advocated. When drains are used, early removal is recommended. There is not enough evidence regarding the type of drain. A new randomized controlled study is currently underway which aims to compare the closed suction drain *vs* the passive closed gravity drain.

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INTRODUCTION

High morbidity is a continuing concern in modern pancreatic surgery, with postoperative pancreatic fistula (POPF) being the most ominous complication[1-3]. POPF is not a life-threatening condition in most cases, but nevertheless, it prolongs the hospital stay, increases the cost of the treatment, and delays adjuvant therapy in malignant disease[4]. The rate of POPF is reported to be in the range of 10%-30% in the majority of papers[1,2,5]. As POPF has significant clinical and economic consequences, attention has focused on lowering the POPF rate.

Several methods have been studied in the past in order to lower the pancreatic fistula rate, including pharmacological prophylaxis with octreotide[6,7] and various technical modifications of pancreatic remnant management after pancreaticoduodenectomy (PD)[8] and after distal pancreatectomy (DP)[9,10]. However, the use of octreotide remains controversial, and none of the studied techniques proved to be superior.

In recent years, the issue of placement and management of intra-abdominal drains following pancreatic resection has attracted attention and is currently widely discussed[11-17]. The placement of prophylactic intra-abdominal drains has been common practice since the 19th century. The rationale for inserting intra-abdominal drains following resection was that the drains were thought to evacuate blood, bile, pancreatic juice and other fluids that may accumulate after surgery[18]. The drains were also thought to allow for early identification of postoperative complications, such as anastomotic dehiscence or early hemorrhage. Moreover, prophylactic intra-abdominal drainage was supposed to avoid the need for additional interventions for intra-abdominal collections by creating a controlled pancreatic fistula[18-24].

However, the controversy over drain placement in acute as well as elective surgeries has persisted since the beginning of modern surgery[25]. Many recent studies show that the use of drains might not be beneficial for patients after abdominal surgery (appendectomy, cholecystectomy, hepatectomy, colectomy, gastrectomy)[26-31]. In fact, the use of drains might be even harmful for the patient, as they can slow down recovery and the restoration of bowel movements, and further prolong the hospital stay; drains may even cause postoperative complications such as retrograde intra-abdominal infection, and hollow organ perforation[15,30]. This might be the result of an artificial access to the peritoneal cavity, the inflammatory response to the drain as a foreign body, increased pain due to the drain, or the loss of fluid and electrolytes[20]. The standard use of drains also interferes with attempts to accelerate recovery through ERAS (enhanced recovery after surgery) programs[32].

Pancreatic surgery is different from the surgery of hollow organs[20]. In contrast to enteric anastomosis dehiscence, which often presents with pneumoperitoneum and frequently causes peritonitis[33], a pancreatic leak is more frequent, but the clinical course is not usually as dramatic[2]. Pancreatic leak or pancreatic fistula can be easily diagnosed by analyzing the amylase concentration in the drain effluent[34]. However, the amylase concentration is increased in the majority of patients on the first postoperative day, even in those patients who will not develop a pancreatic fistula in their postoperative course; this implies that, in the majority of patients, the pancreatic anastomosis is not "water-tight"[35].

Management of intra-abdominal drains has become an important issue in modern pancreatic surgery, as previous studies found that the management of intra-abdominal drains can influence the rate of postoperative complications[18,20,36-38]. Recent systematic reviews and meta-analyses have focused on the routine usage of drains following elective pancreatic resection[20-22,24]. However, there are additional issues to address regarding the use of intra-abdominal drainage following pancreatic surgery, such as the timing of drain removal and the type of drain.

For this reason we carried out a systematic review of studies dealing with all aspects of drain management in pancreatic surgery.

MATERIALS AND METHODS

Search strategy and study selection

We searched the Cochrane Central Registry of Controlled Trials, EMBASE, Web of Science, and PubMed (MEDLINE) for relevant articles published from January 1990 to December 2014. The search was performed independently by two authors (FC and ML) using the terms: "Pancreatectomy", "Drain", "Pancreatic fistula", "Pancreas", and "Postoperative complication". The full search strategy is shown in the Supplementary Appendix (Literature search).

The reference lists of relevant studies were screened to retrieve any further potential studies. No unpublished data or data from abstracts were encountered or used. No language restriction was applied to the search. Abstracts of all potentially relevant articles were read and assessed. All original papers studying the management of drains in pancreatic surgery were retrieved and included in the systematic review.

Inclusion and exclusion criteria

We included articles that reported clinical studies on human subjects with any type of elective pancreatic resection and that compared various strategies of intra-abdominal drain management: *e.g*., drain *vs* no-drain, selective drain use, early *vs* late drain removal, and the use of different types of drains. Studies reporting on drainage for acute pancreatitis were excluded. Studies were included irrespective of their design (prospective/retrospective, randomized controlled, non-randomized controlled, cohort studies/case-control studies) or the length of follow-up. Congress abstracts and personal communications were not considered.

Statistical analysis

All data from selected studies were analyzed independently by two reviewers (Čečka F and Loveček M). We extracted data on methodology, population, interventions including types of drains, outcome measures including POPF rate[39], postoperative morbidity and mortality. Missing data were obtained from the corresponding authors of the studies. Disagreements were resolved in group discussions. Our methodology followed the standard guidelines outlined in the Cochrane Handbook for Systematic Reviews of Interventions[40] and the PRISMA statement (Preferred Reporting Items for Systematic Reviews and Meta-Analyses)[41].

RESULTS

The initial search strategy retrieved 930 publications. Of these, 868 were excluded in the primary selection based on the title and abstract revision (not relevant, not dealing with drain management) and 43 were excluded in the secondary selection after reading the full-text of the potentially relevant studies. Subsequently, the reference lists of all reviewed articles were checked manually; however, this did not lead to identification of any additional studies. Nineteen studies were identified and included in the systematic review, representing a total of 4194 patients (samples ranging from 22 to 1122)[11-17,36-38,42-50]. The reviewers reached agreement on the application of the eligibility criteria for study selection. A flowchart of the literature search strategy according to the PRISMA statement is shown in Figure 1. Only three studies were randomized: published in 2001[13] (n = 179), in 2010[37] (n = 114), and in 2014[17] (n = 137 patients); the last study was the only multi-center study, and the first two were single-center studies. Except for the three randomized studies, all other studies were either retrospective observational, time-cohort or pilot studies.

Table 1 lists the studies analyzing the outcomes of pancreatic resections without drains. A retrospective study comparing two cohorts was published by the group from Memorial Sloan-Kettering Cancer Center (MSKCC) in New York[43]. The authors reported comparable results in both groups (comparable postoperative complications, POPF rate, CT-guided drainage, and length of hospital stay). The only difference was shorter operating time in the group of patients without drains (P = 0.0001). The same group from MSKCC conducted a RCT of drain *vs* no-drain following pancreatic resection[13]. In this trial, the authors described an equal rate of postoperative complications in both groups. However, patients with drain had a higher rate of intra-abdominal collections and fistulas (22% *vs* 9%, P < 0.02) and a higher rate of POPF itself.

Another trial from this department was published in 2013[14]. Six high-volume surgeons were paired according to their operative drainage practices into routine drainers, selective drainers and routine non-drainers. The group of patients with intra-abdominal drainage had a higher POPF rate (P < 0.001) and higher overall morbidity (P = 0.03). However, the patients with drains had significantly higher blood loss in pancreaticoduodenectomy (P < 0.001) as well as in distal pancreatectomy (P < 0.001). Furthermore, the patients in the drained group had longer operating times for both pancreaticoduodenectomy (P < 0.001) and distal pancreatectomy (P < 0.001). The most important fact is that mortality was significantly higher in the no-drain group (3% *vs* 1%, P = 0.02)

The study by Paulus *et al*[16] analyzed abdominal drainage following distal pancreatectomy. There were no differences between the groups regarding overall complications (P = 0.91) or intra-abdominal complications (P = 0.58). Estimated blood loss was higher in the drain group (P = 0.0003).

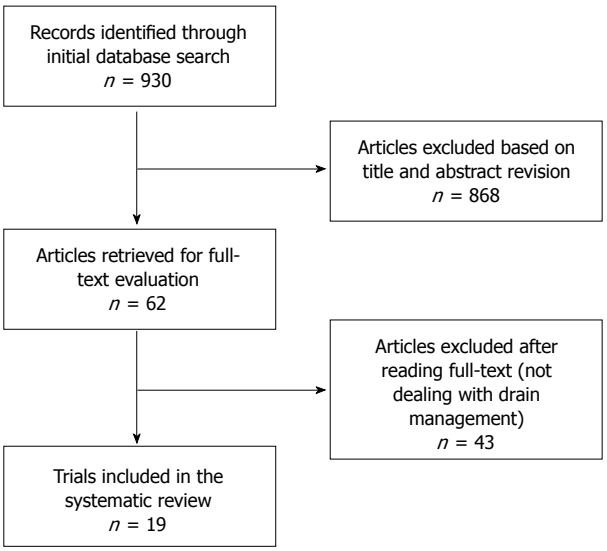

Figure 1 Flowchart of literature search strategy.

Lim *et al*[46] avoided abdominal drainage after uncomplicated pancreaticoduodenectomy in 27 patients at low risk of POPF; these patients were matched to 27 patients undergoing PD with intra-abdominal drainage. Overall morbidity (P = 0.4) and mortality (P = 1) were similar in both groups. The POPF rate (P = 0.009) and hospital stay (P = 0.004) were significantly reduced in the no drainage group.

Mehta *et al*[47] analyzed 709 patients undergoing PD. Compared with the no drain group, patients with a primary drain had a higher overall morbidity (P < 0.001) and POPF rate (P < 0.0001), as well as a longer hospital stay (P = 0.001). Operation time (P = 0.021) and blood loss (P < 0.0001) were significantly higher in the drain-group. It is worth noting that intra-abdominal drainage did not prevent the need for secondary drainage in this study (P = 0.358).

The study by Adham *et al*[11] was retrospective and surgeon-dependent. One surgeon always used an intra-abdominal drain, whereas the second surgeon shifted from using a systematic drain to a no drain policy over the duration of the study. There was no difference in overall complications (P = 0.11), post-pancreatectomy hemorrhage (P = 0.33) or POPF rate (P = 0.34). The requirement for an interventional procedure was equivalent in both groups (P = 0.15).

Behrman *et al*[12] used data from the American College of Surgeons-National Surgical Quality Improvement Program. In this study, 116 patients without drains following distal pancreatectomy were matched using propensity scores with 116 patients with drains. The overall POPF rate (P < 0.01) and overall morbidity (P < 0.05) were more common in patients who received a drain. The placement of a drain did not reduce the need for postoperative interventional procedures (0.29).

Kunstman *et al*[45] calculated FRS (fistula risk score) for 265 patients, 259 of whom were managed without operative drains. The authors reported an unusually high rate of postoperative morbidity (83%) with an acceptable clinically relevant postoperative pancreatic fistula (CR-POPF) rate (8%). The authors concluded that the FRS reliably predicts the absence of CR-POPF in low risk patients and provides an objective way to characterize POPF risk[45].

A time cohort study was published by Fisher[15]. Morbidity (P = 0.02) and POPF (P < 0.0001) were higher in patients with drains. However, postoperative percutaneous drainage (P = 0.001) and readmission rates (P = 0.007) were higher in the no drain group. Based on this preliminary experience, the authors conducted a multicenter randomized controlled trial[17]. A total of 752 patients were planned to be included in the study in order to detect any significant difference between the groups. However, the trial was stopped early by the Data Safety Monitoring Board because of excess mortality in the patients undergoing PD without routine intraperitoneal drainage[17]. After 90 d of follow-up, there were 8 deaths (12%) in the no-drain group and only 2 deaths (3%) in the drain group (P = 0.097). There were more intra-abdominal abscesses (P = 0.033) and abdominal fluid collections (P = 0.033) in the no-drain group. The POPF rate in both groups was not significantly different (P = 0.155); however, 14 out of 21 patients in the drain group had asymptomatic POPF grade A. All 20 patients in the drain group had clinically relevant POPF.

Table 2 lists the studies analyzing the timing of drain removal. Balzano *et al*[42] reported a series of 123 patients with DP. The authors preferred cautious drain management, *i.e.*, maintaining the drain until the daily output diminished to 5 mL in 24 h. Thirty-nine out of 42 patients with POPF were discharged home with the drain and maintained it for a mean duration of 36 d. The authors did not compare this approach to early drain removal.

A time cohort study was published by Kawai *et al*[38]. In the first period, the drain was removed on postoperative day (POD) 8, whereas in the second period the drain was removed on POD 4. The POPF rate (P = 0.0038) as well as intra-abdominal infections (P = 0.0003) and infected intra-abdominal collections (P = 0.0079) were significantly lower in the second period. According to the authors, increasing infections occurred around POD 7, with positive cultures of drainage fluid increasing to 31% on POD 7. This suggests that prolonged placement of a drain might be a major cause of postoperative infectious intra-abdominal complications[38].

Bassi *et al*[37] published a RCT comparing early drain removal (POD 3) *vs* late drain removal (after POD 5) in patients at low risk for POPF. Patients with a high risk of POPF development (amylase value ⩾ 5000 U/L on POD 1) were excluded. Early drain removal was associated with a decreased POPF rate (P = 0.007), abdominal complications (P = 0.002), and pulmonary

Table 1 Main characteristics and results of studies comparing drain *vs* no drain, or selective drain use *n* (%)

Ref.	Year	Type of study	Type of resection, *n*	Type of drain	Groups, *n*	Age (yr), range	POPF	Morbidity	Reoperation	Operation time (min)	Blood loss (mL)	Hospital stay (d)
Jeekel *et al*[44]	1992	Pilot study	PD 22	NA	No drain *n* = 22	43-79	NA	5 (23)	1 (5)	NA	NA	NA
Heslin *et al*[43]	1998	Retrospective	PD 89	Closed-suction	Drain *n* = 51	65 ± 2[1]	3 (6)	23 (45)	2 (4)	386 ± 20[1]	1100 ± 10[1]	12 ± 1[1]
					No drain *n* = 38	65 ± 2[1]	1 (3)	15 (39)	3 (8)	292 ± 13[1]	1100 ± 10[1]	12 ± 1[1]
Conlon *et al*[13]	2001	RCT	PD 139/DP 40	Closed-suction (Jackson-Pratt)	Drain *n* = 88	66 (23-81)[2]	11 (13)	55 (63)	8 (9)	330/190	800/600	9 +
					No drain *n* = 91	69 (33-87)[2]	0 (0)	52 (57)	4 (4)	329/180	800/500	9 +
Fischer *et al*[15]	2011	Time cohort	PD 153, DP 73	Closed-suction	Drain *n* = 179	63 (53-72)[3]	79 (44)	117 (65)	8 (4)	401 (310-490)[3]	400 (200-700)[3]	7 (7-10)[3]
					No drain *n* = 47	59 (51-70)[3]	5 (11)	22 (47)	0 (0)	400 (314-458)[3]	250 (150-500)[3]	7 (6-8)[3]
Paulus *et al*[16]	2012	Retrospective	DP 69	Closed-suction (Jackson-Pratt or Blake)	Drain *n* = 39	52 (44-66)[2]	6 (15)	15 (50)	11 (28)	249 (196-290)[2]	450 (300-750)[2]	9 (7-17)[2]
					No drain *n* = 30	58 (52-68)[2]	0 (0)	20 (51)	8 (27)	195 (176-260)[2]	200 (100-300)[2]	6.5 (5-8)[2]
Lim *et al*[46]	2013	Selective	PD 54	Multichannel open silicone drain	Drain *n* = 27	62 (40-76)[2]	6 (22)	19 (70)	2 (7)	300 (180-540)[2]	400 (50-2000)[2]	15 (11-56)[2]
					No drain *n* = 27	62 (38-78)[2]	0 (0)	15 (56)	1 (4)	270 (170-420)[2]	300 (100-2000)[2]	10 (8-26)[2]
Mehta *et al*[47]	2013	Retrospective	709 PD	Closed-suction (Jackson-Pratt or Blake)	Drain *n* = 251	60[4]	61 (24)	171 (68)	14 (6)	294[4]	572[4]	13.8[4]
					No drain *n* = 458	62.5[4]	48 (10)	248 (54)	26 (6)	201[4]	282[4]	11.3[4]
Adham *et al*[11]	2013	Retrospective	148 PD, 66 DP, 20 CPR, 8 E	Closed-suction (shirley)	Drain *n* = 130	61.5 (20-85)[2]	21 (16)	83 (64)	16 (12)	235 ± 71[1]	471 ± 568[1]	16 (2-98)[2]
					No drain *n* = 112	66.5 (19-85)[2]	14 (13)	45 (67)	17 (15)	265 ± 84[1]	379 ± 387[1]	18 (7-131)[2]
Correa-Gallego *et al*[14]	2013	Retrospective	739 PD, 350 DP, 31 CPR, 2 CSP	Closed-suction (Jackson-Pratt)	Drain PD *n* = 386	65 ± 13[1]	149 (27)	301 (54)	3 (< 1)	295 (250-339)[3]	525 (350-800)[3]	8 (7-11)[3]
					Drain DP *n* = 154					191 (154-229)[3]	400 (200-800)[3]	7 (6-9)[3]
					No drain PD *n* = 353		102 (18)	272 (48)	2 (< 1)	206 (180-247)[3]	400 (250-700)[3]	7 (6-10)[3]
					No drain DP *n* = 196					152 (118-188)[3]	200 (100-400)[3]	5 (5-7)[3]
Behrman *et al*[12]	2014	Propensity-score match cohort	232 DP	NA	Drain *n* = 116	57 +	25 (22)	50 (43)	1 (1)	222 +	NA	6 +
					no draín *n* = 116	59 +	8 (7)	35 (30)	3 (3)	228 +	NA	6 +
Kunstman *et al*[45]	2014	Routine non-drainer	265 PD	NA	Drain *n* = 6	64.2[4]	21 (8)	220 (83)	NA	NA	NA	6 (3-8)[3]
					No drain *n* = 259							
Van Buren *et al*[17]	2014	RCT	137 PD	Closed-suction	Drain *n* = 68	62 ± 12[1]	21 (31)	50 (74)	2 (3)	425 ± 151[1]	460 ± 352[1]	7 (6-9)[3]
					No drain *n* = 69	64 ± 13[1]	14 (20)	52 (75)	6 (9)	407 ± 157[1]	443 ± 344[1]	8 (7-14)[3]

[1]mean ± SD; [2]median (range); [3]median (interquartile range); [4]mean, + median. NA: Not available; RCT: Randomized controlled trial; PD: Pancreaticoduodenectomy; DP: Distal pancreatectomy; CPR: Central pancreatic resection; E: Enucleation; CSP: Central-sparing pancreatectomy.

complications (P = 0.007). The median hospital stay was also shorter in patients with early drain removal (P = 0.018).

Only four studies compared various types of drains following pancreatic resection; these results are described in Table 3.

Aimoto *et al*[36] compared closed-suction drains (Blake) *vs* closed passive drains (Duple) for efficacy in a retrospective study of 33 patients following PD. Only patients with a soft pancreas who developed CR-POPF were included. Overall morbidity was significantly lower in the patients with a Blake drain compared to those with a Duple drain (P < 0.01). The authors concluded that the Blake drains controlled POPF grade B more successfully than did the Duple drains in this study[36].

Schmidt *et al*[48] analyzed the clinical predictors and patient outcomes of pancreatic fistula following PD in

Table 2 Main characteristics and results of studies evaluating timing of drain removal *n* (%)

Ref.	Year	Type of study	Type of resection, *n*	Type of drain	Groups, *n*	Age (yr)	Drain duration (d)	POPF	Morbidity	Reoperation	Op time (min)	Blood loss (mL)	Hospital stay (d)
Balzano *et al*[42]	2005	Retrospective	123 DP	Open silicone 28 CH drain	123	59 (19-85)[2]	36 ± 17 ++	42 (34)	60 (49)	5 (4)	246 ± 87[1]	635 ± 523[1]	11.8 ± 6.1[1]
Kawai *et al*[38]	2006	Time cohort	104 PD	10-mm Penrose (silicon multitubular flat drain)	52 early	66 ± 10[1]	POD 4	2 (4)	19 (37)	0	407 ± 76[1]	1270 ± 1220[1]	42 ± 13[1]
					52 late	67 ± 10[1]	POD 8	12 (23)	35 (67)	0	383 ± 59[1]	1287 ± 1374[1]	35 ± 25[1]
Bassi *et al*[37]	2010	RCT	75 PD, 39 DP	Flat penrose drain 12 mm	57 early	56 ± 14[1]	POD 3	1 (2)	22 (39)	0	285 ± 97[1]	NA	8.7 ± 4[1]
					57 late	57 ± 13[1]	POD after 5	15 (26)	35 (61)	1 (2)	291 ± 86[1]	NA	10.8 ± 6.9[1]

[1]mean ± SD; [2]median (range). ++: only patients with POPF. PD: Pancreaticoduodenectomy; DP: Distal pancreatectomy; RCT: Randomized controlled trial.

Table 3 Main characteristics and results of studies comparing different drain types *n* (%)

Ref.	Year	Type of study	Type of resection, *n*	Type of drain	Morbidity	POPF
Aimoto *et al*[36]	2008	Time cohort	33 PD	Duple drain (*n* = 14)	10 (71)	14 (100)
				Blake drain (*n* = 19)	2 (11)	19 (100)
Schmidt *et al*[48]	2009	Retrospective	510 PD	Penrose drain (*n* = 241)	241 (47)	8 (3)
				Closed-suction (*n* = 269)	269 (53)	38 (14)
Yoshikawa *et al*[49]	2011	Time cohort	97 DP	Penrose drain (*n* = 56)	56 (58)	40 (71)
				Closed-suction (*n* = 41)	41 (42)	26 (63)
Yui *et al*[50]	2014	Time cohort	109 DP	Penrose drain (*n* = 52)	28 (54)	22 (42)
				Closed-suction (*n* = 57)	25 (44)	15 (26)

PD: Pancreaticoduodenectomy; DP: Distal pancreatectomy.

510 patients over a period of 23 years. The authors compared patients with closed-suction drains *vs* open Penrose drains. There was a significantly higher POPF rate in patients with closed-suction drains compared to passive Penrose drains ($P < 0.001$). However, the comparison of drain types was not the primary end-point of this study.

Yoshikawa *et al*[49] studied 97 patients undergoing distal pancreatectomy. In the first period, the authors used Penrose drains, and closed suction drains were used in the second period. The authors stated that closed-suction drains tended to reduce the persistent drainage period and significantly shorten the postoperative stay; however, no exact data were reported.

Yui *et al*[50] described a retrospective comparison of two cohorts of patients undergoing distal pancreatectomy after introducing a new policy for peri-and post-operative management. This new policy included the use of ultrasonically activated scissors, early drain removal and a different type of drain (two open Penrose drains *vs* one closed suction drain). Because several factors changed at the same time, the contribution of each factor remains unclear.

DISCUSSION

This systematic review aimed to evaluate the current knowledge about drain management following pancreatic resection. This topic has been divided into three issues: (1) whether to use routine intra-abdominal drains at all; (2) when to remove the drains; and (3) what type of drain is preferred.

Our review is based on a comprehensive literature search and systematic data aggregation. Nineteen studies met the inclusion criteria. Drain management following pancreatic resection has attracted much attention, especially in the past five years; most of the studies in this review have been published within this period. The first systematic review and meta-analysis assessing drain management was published in 2011; this analyzed the results of the first 4 studies[13,37,38,43]. Diener *et al*[18] included two studies reporting the result of drain omission and two studies analyzing the timing of drain removal. The authors concluded that the evidence is still unclear and that a treatment recommendation could not be made. Other studies have been published since that time, and progress has been made to date[18-24].

Although surgical drains had previously been considered as mandatory following pancreatic resection, a new approach to pancreatic resection emerged without the necessity for intra-abdominal drain insertion with the pilot study by Jeekel *et al*[44], who described 22 cases of pancreaticoduodenectomy without drains. The authors concluded that intra-abdominal drainage did not improve the results of pancreatic resection, and thus, it should not be

considered mandatory. A number of studies have been published since the first pilot study; the majority of the studies were retrospective[11,14,16,43,47] or time cohort[15] in design. These studies showed data suggesting that pancreatic resection can be safely performed without routine drainage; they described either comparable results regarding postoperative morbidity and POPF rate in both groups[11,16,43] or even superior results without a drain[12,14,15,47]. These retrospective observational trials are inevitably subject to selection bias or bias due to the uneven distribution of the involved surgeons' expertise among treatment groups. These studies described higher estimated blood loss or longer operating times in the drain group[14,16,43,47], which suggest that these cases were more difficult or demanding, with a higher risk of postoperative complications regardless of the use of intra-abdominal drains[51]. Another source of bias is the surgeon's preference. In the study by Paulus, only one of three surgeons was responsible for those patients who did not receive a drain[16]. In the study by Adham, one surgeon always used an abdominal drain, while the second surgeon shifted from systematically using a drain to a no-drain policy over the duration of the study[11]. In the study by Correa-Gallego, the six high-volume surgeons involved in the trial were paired according to their operative drainage practices into routine drainers (operative drains placed in over 95% of cases), selective drainers (drains placed in 50% of cases) and routine non-drainers (drains placed in less than 15% of cases)[14].

Such selection bias is excluded in the randomization process in RCTs[13,17]. The first RCT by Conlon showed an equal rate of morbidity in both groups but a higher rate of POPF in the drain group[13]. The last RCT by Van Buren *et al*[17] seems to be in direct contrast to the previous RCT as well as the observational retrospective cohorts.

The Van Buren group had planned to test the hypothesis that "abandoning routine drainage following pancreatic resection would not increase the incidence or severity of postoperative morbidity or mortality". This study was conducted in 9 high-volume academic pancreatic surgery centers and planned to involve 752 patients. However, the Data Safety Monitoring Board stopped the study because of excess mortality in the patients without drainage (12% *vs* 3%, $P = 0.097$)[17]. PD without drain was associated with increased morbidity and clinically relevant POPF in this study.

The differences between the results can be explained in several ways. The multicenter approach seems to provide more validity and generalizability of results[51]. Furthermore, the authors from Memorial Sloan Kettering Cancer Center (MSKCC) did not use what are the currently generally accepted definitions of postoperative morbidity and POPF.

Further analysis of the POPF rate in the multicenter study by Van Buren shows that the overall POPF rate was higher in the drain group, although not significantly so (31% *vs* 20%, $P = 0.155$). However, clinically relevant POPF was higher in the no-drain group (10% *vs* 20%, $P = 0.104$). It is apparent from the results that all of the patients in the group without drains had symptomatic fistula. One hypothesis states that some of the patients would have had asymptomatic fistula if they had had an intra-abdominal drain. In high-risk patients who have a leak from the pancreaticojejunal anastomosis, any excess pancreatic juice is removed *via* the intra-abdominal drain if it is present. After a minor leak has healed, the intra-abdominal drain is removed, and an asymptomatic POPF grade A is classified in the patient. However, in patients with no drain, the pancreatic juice would congest in the retroperitoneum and peripancreatic area with subsequent complications. Subsequent digestion and destruction of the surrounding tissue may be followed by the development of peripancreatic fluid collections, intra-abdominal or retroperitoneal abscesses, delayed gastric emptying, and postoperative hemorrhage.

The controversy regarding the routine abandonment of drains following pancreatic resection is also evident in the study by Correa-Gallego *et al*[14]. Even at the MSKCC, where a RCT[13] showed that routine intra-abdominal drains could be abandoned, twelve years later two out of 6 high-volume pancreatic surgeons still routinely use intra-abdominal drains and two other surgeons drain selectively.

Even though the retrospective observational trials carry a significant risk of selection bias, all of them uniformly suggest that routine abandonment of drains can be safely performed at least in a subset of patients[14,16,43,47]. Therefore, a selective approach to drain placement according to the individual risk-benefit assessment was recommended by some authors[19]. Drains should be placed in high risk patients, whereas they can be omitted in low risk patients[19]. This approach was also adopted in the study by Lim *et al*[46]. They established a predictive model of POPF based on body mass index (BMI), pathologic grading of fatty infiltration, and fibrosis in the pancreatic transection margin. Intra-abdominal drainage was avoided in patients at low risk of POPF after uncomplicated PD. The POPF rate ($P = 0.009$) and hospital stay ($P = 0.004$) were significantly reduced in the no-drainage group. It is not clear whether better results in the no-drain group were due to the avoidance of drainage or due to a lower risk of POPF in the patients[46].

A useful model for determining the risk of POPF is the fistula risk score (FRS)[52]. A simple 10-point FRS based on pancreatic gland texture, certain pathology, pancreatic duct diameter and intraoperative blood loss accurately predicts subsequent CR-POPF.

The fistula risk score was later calculated by McMillan *et al*[53] for the patients in the multicenter RCT published by Van Buren. This work found no differences between the treatment cohorts in terms of the fistula risk score. The authors concluded that patients with moderate and high risk of CR-POPF

should undergo routine drain placement to ensure optimal treatment of the fistula and its consequences, and in patients with low risk, drain placement should be left to the discretion of the surgeon[53].

It is impossible to determine whether the complications seen with the use of drains are because of the drains themselves or because of other factors related to the patient or to the disease that increase the rate of complications.

Traditionally, intra-abdominal drains were inserted following pancreatic resection and maintained until the risk of POPF diminished. This meant in most cases maintaining the drains until the daily output had decreased to below 5 mL per 24 h[42]. Keeping the drains for a longer period of time could reduce the patient's comfort; however, some authors believed that this approach could lower the rate of delayed complications[42]. Surgically placed drains are normally removed "at the surgeon's discretion" with no clear specification as to when the drains should be removed. However, with the introduction of fast-track protocols, the need for reducing hospital-stay, and ultimately providing high-quality cost-effective care, more attention has been paid to drain management. Drain management and especially the timing of drain removal are key factors[37,38].

Both studies comparing early *vs* later drain removal showed superior results for early drain removal, even though there were certain flaws in the study designs. The study published by Kawai *et al*[38] was a time-cohort study, which carries significant bias; the study published by Bassi was criticized for analyzing both procedures (pancreaticoduodenectomy and distal pancreatectomy) together, even though the two procedures are different, with different POPF rates[5] and a different course of POPF development[54]. Furthermore, the authors in both studies used flat Penrose drains, which are now considered obsolete[49,55].

Not much attention has been paid to the various types of drains that are used following intra-abdominal surgery[18,51]. Two types of surgical drains exist: open drains and closed drains. Open drains evacuate collected fluid through an artificial catheter inserted into the postoperative wound. Open drains are considered obsolete because of frequent retrograde infection[55]. Closed drainage is believed to reduce the risk of retrograde microbial contamination compared with open drainage[23].

Closed drains include two types: passive gravity drains and closed-suction drains. The majority of authors prefer various modifications of closed suction drains (Jackson-Pratt, Blake, Shirley)[11,13-17,43,47]. However, some surgeons believe that negative pressure might pose potential hazards to the patients[56], increase the risk of pancreatic fistula or lead to delayed hemorrhage[23]. Therefore, passive gravity drains are preferred by some authors[42,46]. Various types of drains have been studied retrospectively in neck dissection[57] and in liver resection[58]; RCTs were conducted to study the types of drains in cholecystectomy[55] and cardiac surgery[59]. The situation in pancreatic surgery is different, as the pancreatico-enteric anastomosis is not water-tight in most cases, as indicated by an increased amylase level on the 1st POD[34,60,61]; more attention must be paid to the choice of drain type in order to decrease the clinically significant POPF rate. Only four studies have compared the various types of drains in pancreatic surgery[36,48-50]; however, two of them[48,49] were retrospective observational, and most importantly, the comparison of the types of drains was not the primary outcome of the studies, and the studies took place over a very long time period. Furthermore, their results are contradictory.

Diener *et al*[18] stated that the role of different types of drains remains unclear. Strobel also noted that the type of drainage is unknown[51]. Some surgeons believe that negative pressure might increase the risk of pancreatic fistula or lead to delayed hemorrhage[16,23]. Furthermore, there are case reports suggesting that closed-suction drains may have caused small bowel perforations[62-64].

Grobmyer *et al*[65] conducted an ex-vivo study comparing various types of closed-suction drains. The authors demonstrated that commonly used closed-suction drains generate vacuum pressure from-75 to-175 mm Hg and that the practice of "stripping" the drain tubing can generate a maximal pressure of-225 mm Hg and significantly higher sustained pressures than the suction bulb alone. This negative pressure may hinder wound healing or even promote the formation of POPF[65].

On the other hand, a study in favor of closed-suction drains was that by Aimoto *et al*[36], which reported that Blake drains controlled grade B POPF more successfully than closed passive Duple drains. The main conservative management of POPF is sufficient control of the fistula by adequate drainage of the enzyme-rich pancreatic fluid.

Furthermore, most of the recent studies analyzing the role of drains in pancreatic surgery used closed-suction drains[11,13-17,43,47].

A new randomized controlled study is currently underway to compare closed suction drainage *vs* passive closed gravity drains in patients undergoing pancreaticoduodenectomy or distal pancreatectomy (DRAPA: DRAins in PAncreatic surgery)[66]. This study is registered at clinicaltrials.gov under the number NCT01988519 and plans to enroll 223 patients. The primary end-point of this study is the rate of POPF occurrence, and the secondary end-point is postoperative morbidity[66].

In conclusion, the postoperative pancreatic fistula remains a significant problem after pancreatic resection. The pancreatico-enteric anastomosis as well as the suture of the pancreatic resection line is not absolutely water-tight, which is proven by increased amylase in the drain fluid from the first postoperative

day. The majority of the fistulas are asymptomatic. The goal of postoperative management including the management of intra-abdominal drains is to decrease the rate of symptomatic pancreatic fistula. The study by Van Buren *et al*[17] proved that routine omission of intra-abdominal drains leads to worse results in terms of increased postoperative mortality. Although many retrospective studies have reported superior results from pancreatic resection without drains, these studies were influenced by selection bias due to their retrospective nature. Current studies do not lead to definitive conclusions, and further studies are needed to clarify this issue. When drains are used, early removal is recommended. The final issue that has not yet been clarified is the preferred type of drain. Only a few retrospective studies have compared the various types of drains. However, the analysis of drain types was not the primary goal in two of them, and their results were contradictory. A prospective randomized trial is ongoing; it aims to compare closed-suction drainage with closed passive gravity drains. This review also emphasizes the importance of well-designed randomized controlled studies, which are least likely to be influenced by bias and thus provide the highest level of evidence.

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COMMENTS

Background

Postoperative pancreatic fistula is the most ominous complication following pancreatic surgery. New methods are being studied in order to reduce the rate and clinical significance of the pancreatic fistula. Manipulation with intra-abdominal drains is considered to be one of the important measures.

Research frontiers

This review discusses three important issues regarding drain management: (1) whether to use routine intra-abdominal drains at all; (2) when to remove the drains; and (3) what type of drain is preferred.

Innovations and breakthroughs

Most other reviews and meta-analyses have focused only on the question of routine usage or elimination of intra-abdominal drains following pancreatic surgery. However, there are additional issues to address regarding the drains, such as timing of drain removal or the type of drain. This systematic review addresses all aspects of drain management in pancreatic surgery.

Applications

Although many retrospective studies have reported superior results from pancreatic resection without drains, these studies were influenced by selection bias due to their retrospective nature. Current studies do not lead to definitive conclusions, and therefore, further studies are needed to clarify this issue. When drains are used, early removal is recommended in patients at low risk of pancreatic fistula. The final issue that has not yet been clarified is the preferred type of drain. A prospective randomized trial is ongoing that aims to compare closed-suction drainage with closed passive gravity drains.

Terminology

Pancreatic resections are highly invasive surgical procedures that carry significant morbidity. Prophylactic intra-abdominal drains were traditionally considered to help to reduce postoperative complications.

Peer-review

This is a good systematic review about intra-abdominal drainage following pancreatic resection according to the PRISMA guidelines. This study analyzed the outcomes of pancreatic resection with and without intra-abdominal drains, comparing early *vs* late drain removal and analyzing different types of drains.

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SYSTEMATIC REVIEWS

Are faecal markers good indicators of mucosal healing in inflammatory bowel disease?

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Abstract

AIM: To review the published literature concerning the accuracy of faecal inflammatory markers for identifying mucosal healing.

METHODS: Bibliographical searches were performed in MEDLINE electronic database up to February 2015, using the following terms: "inflammatory bowel disease", "Crohn´s disease", "ulcerative colitis", "faecal markers", "calprotectin", "lactoferrin", "S100A12", "endoscop*", "mucosal healing", "remission". In addition, relevant references from these studies were also included. Data were extracted from the published papers including odds ratios with 95%CI, *P* values and correlation coefficients. Data were grouped together according to each faecal marker, Crohn's disease or ulcerative colitis, and paediatric compared with adult study populations. Studies included in this review assessed mucosal inflammation by endoscopic and/or histological means and compared these findings to faecal marker concentrations in inflammatory bowel diseases (IBD) patient cohorts. Articles had to be published between 1990 and February 2015 and written in English. Papers excluded from the review were those where the faecal biomarker concentration was compared between patients with IBD and controls or other disease groups, those where serum biomarkers were used, those with a heterogeneous study population and those only assessing post-operative disease.

RESULTS: The available studies show that faecal markers, such as calprotectin and lactoferrin, are promising non-invasive indicators of mucosal healing. However, due to wide variability in study design, especially with regard to the definition of mucosal healing and evaluation of marker cut offs, the available data do not yet indicate the optimal roles of these markers. Thirty-six studies published between 1990 and 2014 were included. Studies comprised variable numbers of study participants, considered CD (15-164 participants) or UC (12-152 participants) separately or as a combined group (11-252 participants). Eight reports included paediatric patients. Several indices were used to document mucosal inflammation, encompassing eleven

endoscopic and eight histologic grading systems. The majority of the available reports focused on faecal calprotectin (33 studies), whilst others assessed faecal lactoferrin (13 studies) and one study assessed S100A12. Across all of the biomarkers, there is a wide range of correlation describing the association between faecal markers and endoscopic disease activity (*r* values ranging from 0.32 to 0.87, *P* values ranging from < 0.0001 to 0.7815). Correlation coefficients are described in almost all studies and are used more commonly than outcome measures such as sensitivity, specificity, PPV and/or NPV. Overall, the studies that have evaluated faecal calprotectin and/or faecal lactoferrin and their relationship with endoscopic disease activity show inconsistent results.

CONCLUSION: Future studies should report the results of faecal inflammatory markers in the context of mucosal healing with clear validated cut offs.

Key words: Crohn's disease; Ulcerative colitis; Mucosal healing; Faecal calprotectin; Inflammatory bowel disease; Faecal lactoferrin

Core tip: With regard to mucosal inflammation and response to therapy in Crohn's disease and ulcerative colitis patients, mucosal healing may be a more reliable target for treatment than clinical and biochemical assessment. The available studies in this review show that faecal biomarkers are promising non-invasive indicators of mucosal healing and they could be an appropriate surrogate to endoscopy (the gold standard) in inflammatory bowel diseases patients. However, due to a wide variability in the use of clinical indices and marker cut offs, it's difficult to compare their performances. Moreover, a clear definition of mucosal healing is needed.

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INTRODUCTION

The inflammatory bowel diseases (IBD), Crohn's disease (CD) and ulcerative colitis (UC), are chronic diseases characterised by inflammatory changes in the gastrointestinal tract, which can present at any age and are defined according to disease location, extent and histological findings[1]. IBD features chronic inflammatory changes, with a relapsing/remitting course. Symptoms of active disease typically include abdominal pain, diarrhoea, haematochezia and nutritional compromise. Although predominantly involving the gastrointestinal tract, extra-intestinal manifestations such as skin lesions, joint changes and hepatobiliary disease, may be seen in both CD and UC.

Historically, the treatment goal of IBD has been symptom palliation with clinical remission or response used as the primary outcomes in clinical trials and for registration by regulatory bodies such as the FDA. Clinical disease indices such as the Simple Clinical Colitis Activity Index (SCCAI) are examples of indices used in this way, while composite indices such as the CD activity index (CDAI) use a combination of clinical and laboratory data[2,3]. While this approach has many merits, emerging data suggest that other goals may be associated with an improved prognosis.

Mucosal healing (MH) is associated with improved outcomes in clinical trials and has been suggested as the gold standard for remission as it is a direct measure of inflammation of the target organ[4]. In clinical trials of biological drugs, MH has been associated with a lower risk of hospitalisation and colectomy[5], improved symptom control and reduced corticosteroid use[6], and a reduced risk of clinical and surgical relapse following ileocolic resection in patients with CD[7]. Despite much discussion concerning MH as a treatment goal, there is not yet a clear consensus on its definition[8]. In addition to MH, the concept of deep remission (encompassing both clinical remission and mucosal healing) has been developed although is yet to be formally tested in clinical trials[9].

Recently a working group of the International Organisation for the study of Inflammatory Bowel Diseases (IOIBD) published a detailed description of potential targets for the management of IBD. The process leading to a "treat to target" approach in IBD has mirrored that seen in other diseases where tight disease control has led to improved patient outcomes[10]. For both CD and UC, a key target identified is mucosal healing, in addition to the absence of clinical symptoms. While biochemical markers of inflammation in blood (*e.g.*, C-reactive protein (CRP)) and stool (*e.g.*, faecal calprotectin (FC)) were thought to be adjuvant targets, it was concluded that insufficient data exist for them to be used as treatment targets in their own right. At present, the assessment of MH requires ileocolonoscopy[11]. While ileocolonoscopy is the gold standard in assessing the severity and extent of mucosal inflammation and healing in individuals with IBD[12], it is invasive, expensive and, therefore, not appropriate for repeated regular assessment of disease activity.

Faecal tests of inflammation have significant promise as non-invasive biomarkers that may reflect intestinal inflammation. These proteins can be measured easily in a single stool sample and efficiently quantified by enzyme-linked immunosorbent assay (ELISA). Furthermore, a number of these proteins can be measured using point of care devices

facilitating rapid clinical decision-making based on the current inflammatory burden[13]. Recent studies have considered the potential of those non-invasive markers as ways to assist in the diagnosis of IBD and as indicators of the response to therapy[14]. However, for faecal biomarkers to have a key role in the management of IBD in the treat to target era, it is essential that there are robust, accurate and validated data to support specific cut-off values to aid clinical decision making.

This review aims to examine studies that assess mucosal healing by non-invasive faecal tests. The role of several faecal markers will be discussed with comparison to endoscopic assessment.

MATERIALS AND METHODS

Bibliographical searches were performed in MEDLINE electronic database up to February 2015, using the following terms: "inflammatory bowel disease", "Crohn's disease", "ulcerative colitis", "faecal markers", "calprotectin", "lactoferrin", "S100A12", "endoscop*", "mucosal healing", "remission". In addition, relevant references from these studies were also included.

Studies included in this review assessed mucosal inflammation by endoscopic and/or histological means and compared these findings to faecal marker concentrations in IBD patient cohorts. Articles had to be published between 1990 and February 2014 and written in English. Papers excluded from the review were those where the faecal biomarker concentration was compared between patients with IBD and controls or other disease groups (*e.g.*, irritable bowel syndrome), those where serum biomarkers were used, those with a heterogeneous study population and those only assessing post-operative disease. No specific funding was obtained for this study.

RESULTS

Research design

Thirty-six studies published between 1990 and 2014 were included[15-49] Summaries of the studies are shown in Tables 1-6. Studies comprised variable numbers of study participants, considered CD (15 to 164 participants)[15-26,28,38,41-43,45,46,50,51] or UC (12 to 152 participants)[16,22,23,26,28,35-37,39,42,45-48,50,52] separately or as a combined group (11 to 252 participants)[22,23,28-34,40,42,44,50]. Eight reports included paediatric patients[16,31,34,35,38,43,44,50].

Several indices were used to document mucosal inflammation, encompassing eleven endoscopic and eight histologic grading systems. Some of these systems have been validated (*e.g.,* CDEIS and SES-CD), whilst others utilised grading systems that have not been widely validated. The majority of the available reports focused on faecal calprotectin (33 studies)[15-37,42-44,50], whilst others assessed faecal lactoferrin (13 studies)[17-20,22-24,26,28,30,32,38] and one study assessed S100A12[42]. In addition, studies utilised different faecal biomarker concentration cut offs for the assessment of markers and scoring systems.

Across all of the biomarkers, there is a wide range of correlation describing the association between faecal markers and endoscopic disease activity (*r*-values ranging from 0.32 to 0.87, *P*-values ranging from < 0.0001 to 0.7815). Correlation coefficients are described in almost all studies and are used more commonly than outcome measures such as sensitivity, specificity, PPV and/or NPV. Overall, the studies that have evaluated faecal calprotectin (FC) and/or faecal lactoferrin (FL) and their relationship with endoscopic disease activity show inconsistent results (Tables 1 and 2). Fewer studies have studied the correlation between FC and FL with histologic severity (Tables 4 and 5).

Faecal calprotectin and endoscopic severity

Of the 28 studies investigating the ability of FC to determine endoscopic disease activity in patients with IBD (Table 1), 17 specifically included patients with CD. Two reports demonstrate high sensitivity and specificity[15,21]. However, the number of patients in these studies was relatively low (n = 64 and 122, respectively). In a cohort of 64 CD patients, Schoepfer and colleagues used a FC cut off concentration of 70 μg/g to demonstrate a sensitivity and specificity of 89% and 72% for the identification of MH, respectively[21]. On the other hand, in a cohort of 122 CD patients, af Björkesten *et al*[15] found a sensitivity of 84% and a specificity of 74% with a FC cut off of 94 μg/g. While these values are comparable, the studies used different SES-CD scores to reflect endoscopic remission (SES-CD $\leqslant$ 3 and $\leqslant$ 2 respectively). Reanalysis using a SES-CD score of 0 (absence of ulcers) in Björkesten's study reduced the ability of FC to detect remission. In both studies only FC was capable of discriminating between various degrees of disease activity in contrast to other indicators such as CRP and the CDAI.

Where calculated, PPV and NPV are variable between the studies of FC in CD patients. Much of this variability appears to be secondary to differences in the cut off concentrations for the faecal biomarkers and the cut off endoscopic scores used to define MH.

Thirteen studies described the use of FC concentration and its correlation with mucosal inflammation and healing in UC patients (Table 1). For all of these studies there was a statistically significant association between FC concentration and mucosal inflammation. However, only seven of these studies reported sensitivity, specificity, PPV and NPV for their studies with respect to a specific FC cut off concentration[16,23,27,45-48]. The largest study, which included 115 patients with UC, demonstrated a sensitivity of 93%, specificity of 71%, PPV of 91% and NPV of 81% using a FC cut off of 50 μg/g[27]. Re-evaluation of this data using a higher cut off of

Table 1 Studies investigating the correlation between faecal calprotectin concentrations and endoscopic activity in subjects with inflammatory bowel diseases

Ref.	Number of participants		Population	Endoscopic index used	Endoscopic index cut off	Faecal calprotectin cut off (μg/g)	Outcome measures				Correlation	
	CD	UC					Sensitivity	Specificity	PPV	NPV	*r* value	*P* value
Crohn's disease studies												
Falvey *et al*[46]	59		Adults	SES-CD	≤ 3	125	71%	71%	85%	50%	0.55%	< 0.0001
						200	60%	79%	88%	45%		
Lobatón *et al*[51]	85		Adults	CDEIS	< 3	274 ELISA	77%	97%	75%	98%	0.784%	< 0.001
					< 3	272 QPOC	79%	97%	76%	98%	0.722%	< 0.001
					0	262 ELISA	75%	76%				
					0	200 QPOC	75%	77%				
Nancey *et al*[45]	78		Adults	SES-CD	≤ 2	250	71%	78%	79%	71%	0.53%	< 0.0001
						100	88%	38%	62%	73%		
D'Haens *et al*[47]	87		Adults	CDEIS	≤ 3	< 250	94.1%	62.2%	48.5%	96.6%	0.419%	< 0.001
				SES-CD	0	< 250	51.6%	82.6%	89.2%	38%	0.49%	< 0.001
af Björkesten *et al*[15]	64		Adults	SES-CD	≤ 2	< 100	81%	74%	-	-	0.56%	< 0.001
						< 94	84%	74%	-	-		
				SES-CD	0	< 94	82%	78%	-	-		
Aomatsu *et al*[16]	18		Paediatrics	SES-CD	0	100	94.7%	50%	87.8%	71.4%	0.76%	< 0.01
						150	94.7%	50%	87.8%	71.4%		
Sipponen *et al*[17]	19		Adults	SES-CD	≤ 2	< 100	-	80%	-	-	-	-
Schoepfer *et al*[21]	122		Adults	SES-CD	≤ 3	< 50	89%	58%	89%	61%	0.75%	< 0.01
						< 70	89%	72%	88%	76%		
Langhorst *et al*[23]	43		Adults	SES-CD		> 6	100%	30%	82.5%	100%	0.35%	< 0.05
						> 48	81.8%	80%	93.1%	57.1%		
Schoepfer *et al*[22]	36		Adults	SES-CD	≤ 19	50	-	-	-	-	-	< 0.0001
Sipponen *et al*[18]	61		Adults	SES-CD (total)	≤ 3	< 100	-	-	-	-	0.662%	< 0.001
				SES-CD (colon)	≤ 3	< 100	-	-	-	-	0.642%	< 0.001
				SES-CD (ileal)	≤ 3	< 100	-	-	-	-	0.317%	> 0.05
Sipponen *et al*[19]	15		Adults	CDEIS	≤ 2	< 200	87%	100%	100%	70%	0.831%	< 0.001
Sipponen *et al*[20]	77		Adults	CDEIS	≤ 2	< 50	91%	44%	76%	73%	0.729%	< 0.001
						< 100	81%	69%	84%	66%		
						< 200	70%	92%	94%	61%		
Jones *et al*[24]	164		Adults	SES-CD	≤ 6	≤ 50	-	-	-	-	0.45%	< 0.05
Denis *et al*[25]	28		Adults	CDEIS	≤ 5	< 50	-	-	-	-	-	0.57
Schoepfer *et al*[26]	24		Adults	SES-CD	≤ 19	< 50	-	-	-	-	-	0.0001
D'Incà *et al*[28]	31		Adults	SES-CD		> 80	-	-	-	-	0.48%	0.008
Mixed inflammatory bowel disease population studies												
Molander *et al*[29]	183	69	Mixed	SES-CD; Mayo	≤ 2; ≤ 1	< 100	-	-	-	72%	-	< 0.0001
Vieira *et al*[30]	38	40	Adults	CDEIS; Mayo	≤ 2; ≤ 2	> 200.01	88.6%	97.1%	97.5%	86.8%	-	0
Schoepfer *et al*[22]	36	28	Adults	SES-CD; Rachmilewitz	≤ 19; ≤ 4	50	-	-	-	-	-	< 0.0001
Canani *et al*[50]	26	32	Paediatrics	Saverymuttu	≤ 1	143	-	-	-	-	0.46%	≤ 0.05
Fagerberg *et al*[31]	27	10	Paediatrics	Saverymuttu		< 85.7	-	-	-	-	0.65%	< 0.001
Silberer *et al*[32]	21	18	Adults	Stange		18.6	61.5%	95%	-	-	-	< 0.0001
Røseth *et al*[33]	17	28	Adults	Farup		< 50	0%	100%	-	97.8%	-	-
Bunn *et al*[34]	2	9	Paediatrics	Saverymuttu			-	-	-	-	0.65%	< 0.05
Ulcerative colitis studies												
Falvey *et al*[46]		38	Adults	Baron	0	125	74%	80%	85%	67%	0.55%	< 0.0001
						200	58%	95%	95%	59%		
Nancey *et al*[45]		55	Adults	Rachmilewitz	≤ 2	250	91%	87%	87%	91%	0.75%	< 0.0001
						100	100%	53%	85%	100%		
Kristensen *et al*[48]		62	Adults	Mayo	0	61 Cal	84.1%	83.3%	92.5%	68.2%		< 0.001
					0	96 BM	90.9%	83.3%	93%	78.9%		< 0.001
					≤ 1	110 Cal	80%	66.6%	69.2%	78%		
					≤ 1	259 BM	83.3%	71.9%	73.5%	82.1%		
D'Haens *et al*[47]		39	Adults	Mayo	0	< 250	71%	100%	100%	47.1%	0.56%	< 0.001
Komraus *et al*[35]		16	Paediatrics	Rachmilewitz		< 50	-	-	-	-	0.52%	0.0391
Aomatsu *et al*[16]		17	Paediatrics	Matts	≤ 6	100	94.1%	50%	88.9%	66.7%	0.84%	< 0.01
						150	91.2%	87.5%	96.9%	70%		
Schoepfer *et al*[27]		115	Adults	Rachmilewitz	< 4	< 50	93%	71%	91%	81%	0.83%	< 0.001
						< 100	86%	88%	96%	65%		
Langhorst *et al*[23]		42	Adults	Mayo		> 6	100%	6.7%	6.6%	100%	0.49%	< 0.001
						> 48	81.5%	72.3%	84.6%	68.8%		

Schoepfer *et al*[22]	28	Adults	Rachmilewitz	≤ 4	50	-	-	-	-	-	0.0025
Schoepfer *et al*[26]	12	Adults	Rachmilewitz	≤ 1	< 50	-	-	-	-	-	0.0335
D'Incà *et al*[28]	46	Adults	Mayo		> 80	-	-	-	-	0.511%	0.001
Hanai *et al*[36]	31	Adults	Matts	≤ 1		-	-	-	-	0.81%	< 0.001
Røseth *et al*[37]	62	Adults	Sandborn	≤ 1	< 10	-	34%	-	-	0.57%	< 0.0001
					< 20	-	62%	-	-		

CD: Crohn's disease; UC: Ulcerative colitis; PPV: Positive predictive value; NPV: Negative predictive value; SES-CD: Simple endoscopic score for Crohn's disease[54]; CDEIS: Crohn's disease endoscopic index of severity[55]; ELISA: Enzyme linked immunosorbant assay; QPOC: Quantitative point of care test; Mayo: Mayo endoscopic sub-scoring of ulcerative colitis[56]; Rachmilewitz: Rachmilewitz endoscopic score[57]; Saverymuttu: Non-standard endoscopic scoring system[58]; Stange: Non-standard endoscopic scoring system[59,60]; Farup: Non-standard endoscopic scoring system[61]; Baron: Baron score; Matts: Matts score[62]; Sandborn: Non-standard endoscopic scoring system[63]; Cal: Calpro ELISA: Calpro Calprotectin ELISA, Calpro AS, Norway; BM: BM ELISA, EK-CAL, Buhlmann Laboratories AG, Switzerland; Farmer: Non-standard endoscopic scoring system[64]; Faecal Hb: Faecal haemoglobin; PMN-e: Polymorhonuclear elastase; Hb-Hp: Haemoglobin; Haptoglobin complex; D'Haens: Non-standard histologic scoring system[65]; Fazio: Non-standard histologic scoring system[66]; Floren: Non-standard histologic scoring system[67].

Table 2 Studies investigating the correlation between faecal lactoferrin concentrations and endoscopic activity in subjects with inflammatory bowel diseases

Ref.	Number of participants		Population	Endoscopic index used	Endoscopic index cut off	Faecal lactoferrin cut off (μg/mL)	Outcome measures				Correlation	
	CD	UC					Sensitivity	Specificity	PPV	NPV	*r* value	*P* value
Crohn's disease studies												
Sipponen *et al*[17]	19		Adults	SES-CD	≤ 2	< 7.25	-	80%	-	-	-	-
Pfefferkorn *et al*[38]	54		Paediatrics	Unique score		≥ 7.25	100%	43%	70%	100%	-	< 0.001
						≥ 60	84%	74%	81%	77%		
Sipponen *et al*[18]	61		Adults	SES-CD (total)	≤ 3	< 7.25	-	-	-	-	0.705%	< 0.001
				SES-CD (colon)	≤ 3	< 7.25	-	-	-	-	0.627%	< 0.001
				SES-CD (ileal)	≤ 3	< 7.25	-	-	-	-	0.18%	> 0.05
Sipponen *et al*[19]	15		Adults	CDEIS	≤ 2	< 10	77%	100%	100%	58%	0.865%	< 0.001
Sipponen *et al*[20]	77		Adults	CDEIS	≤ 2	< 10	66%	92%	94%	59%	0.773%	< 0.001
						< 7.25	71%	83%	89%	60%		
Jones *et al*[24]	164		Adults	SES-CD	≤ 6	< 7.25	-	-	-	-	0.48%	< 0.05
Langhorst *et al*[23]	43		Adults	SES-CD		> 7.25	81.8%	60%	87.1%	50%	0.42%	< 0.01
						> 7.05	81.8%	60%	87.1%	50%		
Schoepfer *et al*[22]	36		Adults	SES-CD	≤ 19	7	-	-	-	-	-	< 0.0001
Schoepfer *et al*[26]	24		Adults	SES-CD	≤ 19	< 7	-	-	-	-	-	0.0008
D'Incà *et al*[28]	31		Adults	SES-CD			-	-	-	-	0.192%	0.545
Mixed inflammatory bowel disease population studies												
Vieira *et al*[30]	38	40	Adults	CDEIS; Mayo	≤ 2; ≤ 2	4-8	93.2%	76.5%	83.7%	89.7%	-	0
Schoepfer *et al*[22]	36	28	Adults	SES-CD; Rachmilewitz	≤ 19; ≤ 4	7	-	-	-	-	-	< 0.0001
Silberer *et al*[32]	21	18	Adults	Stange		6.64	33.3%	95%	-	-	-	0.0059
Ulcerative colitis studies												
Langhorst *et al*[23]		42	Adults	Mayo		> 7.25	88.9%	66.7%	82.8%	76.9%	0.56%	< 0.001
						> 7.05	92.6%	66.7%	83.3%	83.3%		
Schoepfer *et al*[22]		28	Adults	Rachmilewitz	≤ 4	7	-	-	-	-	-	0.078
Schoepfer *et al*[26]		12	Adults	Rachmilewitz	≤ grade 1	< 7	-	-	-	-	-	0.7815
D'Incà *et al*[28]		46	Adults	Mayo			-	-	-	-	0.354%	0.023

CD: Crohn's disease; UC: Ulcerative colitis; PPV: Positive predictive value; NPV: Negative predictive value; SES-CD: Simple endoscopic score for Crohn's disease[54]; CDEIS: Crohn's disease endoscopic index of severity[55]; Mayo: Mayo endoscopic sub-scoring of ulcerative colitis[56]; Rachmilewitz: Rachmilewitz endoscopic score[57]; Stange: Non-standard endoscopic scoring system[59,60].

100ug/g resulted in values of 86%, 88%, 96% and 65%, respectively. The correlation coefficient of r = 0.83 for UC was higher than found in CD patients (r = 0.75). Again, FC was the only marker that was able to discriminate inactive from mild, moderate and highly active disease. A further study evaluating patients with UC using the Mayo Endoscopic Subscore and FC with a cut off of 48 μg/g, determined a sensitivity of 81.5% and specificity of 72.3%[23]. In contrast, an earlier study from the same region reported specificity of only 34% (for FC with cut off of 10 μg/g), or 62% using a cut off of 20 μg/g[37]. Four more recent studies have been more thorough in describing the association between FC concentration and endoscopic remission[45-48], although in relatively modest numbers of patients (38-62 patients only). Kristensen *et al*[48] analysed both Mayo 0 and Mayo 0 and 1 combined for two different commercial FC assays. Not surprisingly, specificity and PPV were greater when using the Mayo 0 score with both FC assays. On the other hand, in a

Table 3 Studies investigating the correlation between other faecal marker concentrations and endoscopic activity in subjects with inflammatory bowel diseases

Ref.	Number of participants		Population	Endoscopic index used	Endoscopic index cut off	Faecal marker measured	Faecal marker cut off	Outcome measures				Correlation	
	CD	UC						Sensitivity	Specificity	PPV	NPV	*r* value	*P* value
Nancey *et al*[45]	78		Adults	SES-CD	≤ 2	Neopterin	200 pmol/g	74	73	73	74	0.47	< 0.001
							150 pmol/g	80	65	68	78		
		55	Adults	Rachmilewitz	≤ 2	Neopterin	200 pmol/g	74	100	100	73	0.72	< 0.001
							150 pmol/g	84	100	100	78		
Nakarai *et al*[39]		152	Mixed	Mayo	0	Faecal Hb	< 100 ng/mL	92	71	37	97	0.5409	< 0.0001
						Faecal Hb	< 60 ng/mL	94	74	40	98		
				Mayo	≤ 1	Faecal Hb	< 100 ng/mL	60	87	85	64		
						Faecal Hb	< 60 ng/mL	58	90	88	64		
Langhorst *et al*[23]	43		Adults	SES-CD		PMN-e	< 0.062 μg/mL	81.8	70	90	54.8	0.32	< 0.05
		42	Adults	Mayo		PMN-e	< 0.062 μg/mL	70.4	66.7	79.2	55.6	0.36	< 0.05
Silberer *et al*[32]	21	18	Adults	Stange		PMN-e	0.124	79.5	95	-	-	-	< 0.0001
						Lysozyme	1.3	47.5	95	-	-	-	< 0.0001
						α1-AT	158	20	95	-	-	-	-
						Faecal Hb	1.8	61.5	95	-	-	-	-
						Hb-Hp	0.8	64.1	95	-	-	-	-
Moran *et al*[40]	7	21	Mixed	Farmer		α1-AT	≤ 0.58 mg/g	-	-	-	-	0.83	0.001
Cellier *et al*[41]	95		Adults	CDEIS		α1-AT		-	-	-	-	0.26	0.001

CD: Crohn's disease; UC: Ulcerative colitis; PPV: Positive predictive value; NPV: Negative predictive value; SES-CD: Simple endoscopic score for Crohn's disease[54]; CDEIS: Crohn's disease endoscopic index of severity[55]; Mayo: Mayo endoscopic sub-scoring of ulcerative colitis[56]; Rachmilewitz: Rachmilewitz endoscopic score[57]; Stange: Non-standard endoscopic scoring system[59,60]; Farmer: Non-standard endoscopic scoring system[64]; Faecal Hb: Faecal haemoglobin; PMN-e: Polymorhonuclear elastase; Hb-Hp: Haemoglobin and Haptoglobin complex.

study of 39 adults with UC, D'Haens *et al*[47] used Mayo 0 and a FC cut off of < 250 μg/g leading to 100% specificity and NPV, but just 50% PPV. Therefore, this would suggest that while a FC concentration of greater than 250 μg/g is highly predictive of the presence of mucosal inflammation in UC, this concentration is no better than flipping a coin for determining whether a patient has mucosal healing.

Faecal calprotectin and histological assessment

FC has been compared with histological activity in only 11 studies (Table 4). A study of 61 CD patients showed a significant correlation between FC concentration (r = 0.563, P < 0.01) and colonic or ileocolonic disease, but not with ileal disease[18]. This is consistent with an earlier study published by the same group[19], which demonstrated a significant association between FC concentration and pretreatment colonic disease (r = 0.522, P = 0.046) although only 15 patients were included.

For UC, the patient groups are small in all of the studies and there are mixed results regarding the correlation between FC concentration and histological appearance. Furthermore, few studies report a FC cut off concentration that optimally reflects MH.

Paediatric studies evaluating faecal calprotectin

Only one of eight paediatric studies documented sensitivity, specificity, PPV and NPV in addition to correlation coefficients[16]. Although the sensitivity was high in both CD (94.7%) and UC patients (94.1%), when utilising a cut off of 100 μg/g, the specificity was only 50%. Using a cut off of 150 μg/g, the specificity for active UC increased to 87.5%. Furthermore, Aomatsu *et al*[16] demonstrated that FC correlates closely with the SES-CD and Matt's grading (r = 0.760 and 0.838, respectively) - the strongest correlations identified amongst these studies.

Fagerberg *et al*[43] studied a paediatric group with predominantly colonic CD. Experienced gastrointestinal histopathologists divided the patients into two groups (inflamed and non-inflamed), based upon conventional criteria for IBD. Using a FC cut off of 50 μg/g resulted in sensitivity 95%, specificity 93%, PPV 95% and NPV 93%. In 2007 Fagerberg *et al*[31] evaluated a mixed group of children with CD and UC. Using a cut off of 85.7 μg/g for FC, the authors demonstrated a sensitivity 93%, specificity 82%, PPV 93% and NPV 82% for the identification of mucosal healing.

It is difficult to directly compare paediatric and adult studies of faecal biomarkers due to the heterogeneity of the study designs, particularly with respect to the use of different endoscopic indices and the definition of MH.

Faecal lactoferrin and endoscopic severity

Ten of twelve studies focusing on FL included just patients with CD (Table 2). For example, Langhorst *et al*[20] used the SES-CD to demonstrate a sensitivity of 81.8% and specificity of 60% for FL (r = 0.35, P < 0.05)[23]. In another study using the same cut off concentration (< 7.25 μg/g), a sensitivity of 71% and a specificity 83% were demonstrated with a PPV of 89% and NPV of 60%. A PPV of 100% has been shown in a further report including just 15 patients: however the NPV in this series was only 58%[19].

In a group of patients with UC, Langhorst *et al*[28]

Table 4 Studies investigating the correlation between FC concentrations and histologic activity in subjects with inflammatory bowel diseases

Ref.	Number of participants		Population	Histology index used	Histology index cut off	Faecal calprotectin cut off (μg/g)	Outcome measures				Correlation	
	CD	UC					Sensitivity	Specificity	PPV	NPV	*r* value	*P* value
Crohn's disease studies												
Sipponen *et al*[18]	61		Adult	D'Haens (ileocolonic)		< 100	-	-	-	-	0.563	< 0.01
				D'Haens (ileal)		< 100	-	-	-	-	0.311	> 0.05
Sipponen *et al*[19]	15		Adult	D'Haens (pretreatment colonic)		< 200	-	-	-	-	0.522	0.046
				D'Haens (posttreatment colonic)		< 200	-	-	-	-	-	> 0.05
				D'Haens (ileal)		< 200	-	-	-	-	-	> 0.05
Canani *et al*[50]	26		Paediatric	Saverymuttu	≤ 1	143	-	-	-	-	0.681	< 0.0001
Kaiser *et al*[42]	32		Adult	Unique score	0	< 50	-	-	-	-	0.412	< 0.05
D'Incà *et al*[28]	31		Adult	Fazio		> 80	81%	80%	95%	-	0.117	0.545
Fagerberg *et al*[43]	22		Paediatric	Unique score		< 50	95%	93%	95%	93%	-	< 0.00001
Mixed inflammatory bowel disease population studies												
Vieira *et al*[30]	38	40	Adult	Unique score		> 200	77%	100%	100%	68%	-	0
Canani *et al*[50]	26	32	Paediatric	Saverymuttu	≤ 1	143	94%	64%	81%	87%	0.655	< 0.05
D'Incà *et al*[28]	31	46	Adult	Fazio; Floren (SES-CD; Mayo)	> 80	79	74%	92%	-	-	-	
Fagerberg *et al*[31]	27	10	Paediatric	Saverymuttu	≤ 2	< 50	93%	73%	90%	80%	0.75	< 0.001
						< 85.7	93%	82%	93%	82%		
				Saverymuttu			-	-	-	-	0.79	< 0.001
Kolho *et al*[44]	9	16	Paediatric	Farup		50	-	-	69%	100%	-	-
						100	-	-	72%	96%	-	-
Bunn *et al*[53]	2	9	Paediatric	Saverymuttu	≤ 6	6.3	100%	80%	-	-	0.74	< 0.01
Ulcerative colitis studies												
Canani *et al*[50]		32	Paediatric	Saverymuttu	≤ 1	143	-	-	-	-	0.661	< 0.0001
D'Incà *et al*[28]		46	Adult	Floren		> 80	78%	70%	90%	-	0.323	0.042
Kaiser *et al*[42]		27	Adult	Unique score		< 50	-	-	-	-	0.311	0.14
Røseth *et al*[37]		62	Adult	Farup	≤ 1	< 10	-	50%	-	-	0.70	< 0.0001
						< 20	-	81%	-	-		

CD: Crohn's disease; UC: Ulcerative colitis; PPV: Positive predictive value; NPV: Negative predictive value; SES-CD: Simple endoscopic score for Crohn's disease[54]; Mayo: Mayo endoscopic sub-scoring of ulcerative colitis[56]; Saverymuttu: Non-standard endoscopic scoring system[58]; Farup: Non-standard endoscopic scoring system[61]; D'Haens: Non-standard histologic scoring system[65]; Fazio: Non-standard histologic scoring system[66]; Floren: Non-standard histologic scoring system[67].

used the Mayo Endoscopic Subscore (without a given cut off concentration for FL), leading to specificity and sensitivity of 92.6% and 66.7%, respectively, with a correlation of $r = 0.56$, $P < 0.001$[23]. A different cohort using the same index showed a lower correlation coefficient for FL ($r = 0.354$, $P = 0.023$)[28].

Faecal lactoferrin and histological assessment

Four studies have evaluated correlations between FL and histologic severity[18,19,28,30]. Sipponen *et al*[18] described a significant correlation between FL and colonic or ileocolonic CD (r=0.543), but not for ileal disease ($r = 0.291$)[18]. Subsequently, the same authors divided patients into subgroups of pretreatment colonic, post-treatment colonic and ileal disease[19]. This report did not find a significant correlation between FL and mucosal histology. An additional study performed by D'Incà *et al*[28] with only 15 participants demonstrated moderate sensitivity (77%), specificity (80%) and PPV (95%), which was comparable to the performance of FC in the same group of patients.

Only one study has measured FL and histologic severity in patients with UC. A sensitivity of 75%, a specificity of 60%, a PPV of 87% and a significant correlation ($r = 0.544$) was ascertained in this report[28].

Paediatric studies evaluating faecal lactoferrin

Only one study has assessed FL in children with CD[38]. Using an unvalidated endoscopic grading system, the patients were divided into active and inactive groups. A cut off of 7.25 μg/g demonstrated a sensitivity of 100% and a specificity of 43%, whereas a cut off of 60 μg/g resulted in a lower sensitivity (84%) but higher specificity (74%). Again, it is hard to compare these outcomes to the adult studies evaluating FL due to marked variability in study design.

Other faecal markers

Although the majority of studies included in this review have evaluated FC and FL, other faecal markers have also been assessed including α1-antitrypsin,

Table 5 Studies investigating the correlation between faecal lactoferrin concentrations and histologic activity in subjects with inflammatory bowel diseases

Ref.	Number of participants		Population	Histology index used	Histology index cut off	Faecal lactoferrin cut off (μg/mL)	Outcome measures				Correlation	
	CD	UC					Sensitivity	Specificity	PPV	NPV	*r* value	*P* value
Crohn's disease studies												
Sipponen *et al*[18]	61		Adults	D'Haens (ileocolonic)		< 7.25	-	-	-	-	0.543	< 0.01
				D'Haens (ileal)		< 7.25	-	-	-	-	0.291	> 0.05
Sipponen *et al*[19]	15		Adult	D'Haens (pretreatment colonic)		< 10	-	-	-	-	0.482	0.069
				D'Haens (posttreatment colonic)		< 10	-	-	-	-	-	> 0.05
				D'Haens (ileal)		< 10	-	-	-	-	-	> 0.05
D'Incà *et al*[28]	31		Adult	Fazio			77%	80%	95%	-	0.477	0.009
Mixed inflammatory bowel disease studies												
Vieira *et al*[30]	38	40	Adults	Unique score		4-8	90%	92%	96%	83%	-	-
D'Incà *et al*[28]	31	46	Adults	Fazio; Floren (SES-CD; Mayo)		7	76%	67%	90%	-	-	-
Ulcerative colitis studies												
D'Incà *et al*[28]		46	Adults	Floren		7	75%	60%	87%	92%	0.544	0.0001

CD: Crohn's disease; UC: Ulcerative colitis; PPV: Positive predictive value; NPV: Negative predictive value; SES-CD: Simple endoscopic score for Crohn's disease[55]; Mayo: Mayo endoscopic sub-scoring of ulcerative colitis[57]; D'Haens: Non-standard histologic scoring system[66]; Fazio: Non-standard histologic scoring system[67]; Floren: Non-standard histologic scoring system[68].

polymorphonuclear elastase, lysozyme, faecal haemoglobin (FHb), haemoglobin-haptoglobin complex (Hb-Hp), neopterin and S100A12 (Tables 3 and 6).

Cellier *et al*[41] compared faecal α1-antitrypsin to CDEIS in 121 CD patients and found no correlation (r = 0.26). In contrast, Moran *et al*[68] demonstrated in 28 IBD patients a significant correlation between faecal α1-antitrypsin and an alternative endoscopic index (r = 0.83, P = 0.001)[68].

Faecal polymorphonuclear elastase (PMN-e) is significantly correlated with endoscopic severity in CD (r = 0.32) and UC patients (r = 0.36)[23]. Similar results for a mixed group of patients were found by Silberer *et al*[32].

Nakarai *et al*[39] assessed faecal haemoglobin concentrations in 152 UC patients and compared this with the Mayo Endoscopic Score (threshold of mucosal healing). FHb showed sensitivity 94%, specificity 74%, PPV 40%, and NPV 98%.

Of the studies included in this review, only Kaiser *et al*[42] investigated the faecal marker S100A12. The specificity for both CD and UC subgroups was 100%, whereas the sensitivity was 81% in CD and 91% in UC.

DISCUSSION

Faecal biomarkers such as FC and FL offer tremendous promise as non-invasive markers of mucosal inflammation. As therapeutic targets move from symptom control to mucosal healing, it is imperative that non-invasive markers of inflammation are firstly validated and then become available for routine clinical use. This could allow more regular assessment of inflammation with subsequent timely clinical decisions and possibly lead to a reduced requirement for follow-up endoscopies. Sensitive and specific biomarkers are essential if a true treat-to-target approach is to be adopted.

At best the currently available studies show a mixed picture with few findings strongly replicated across multiple studies. This variability is reflected in diverse study designs with a wide range of endoscopic and other indices employed. Even within studies using the same indices, variable scores have been used to define MH or remission. Additionally, a wide range of cut off concentrations for faecal biomarkers have been used, leading to difficulty in the interpretation of individual results. Until a clear target for treatment is defined, it is difficult to resolve many of the differences between these studies.

Correlation coefficients are a useful means of comparing the association between two sets of continuous data (such as faecal biomarker concentration and mucosal inflammation). However, once such correlations have been shown to be significant, it is essential that accurate cut-off concentrations are determined for biomarkers using categorical data for mucosal inflammation. This allows sensitivity, specificity, positive and negative predictive values (in addition to accuracy) to be determined. These parameters are clinically useful, whereas correlation coefficients provide limited clinical relevance. Unfortunately, few studies provided in depth statistical analysis including all the required parameters.

While there were a large number of studies that assessed the utility of faecal biomarkers in reflecting mucosal inflammation at a single point in time, few followed patients prospectively to determine the prognostic significance of elevated biomarkers. In clinical medicine, such prognostic data are essential in determining appropriate treatment escalation and de-escalation.

Future studies

We suggest a number of ways in which future studies

Table 6 Studies investigating the correlation between other faecal marker concentrations and histologic activity in subjects with inflammatory bowel diseases

Ref.	Number of participants		Population	Histology index used	Histology index cut off	Faecal marker measured	Faecal marker cut off	Outcome measures				Correlation	
	CD	UC						Sensitivity	Specificity	PPV	NPV	*r* value	*P* value
Crohn's disease studies													
Kaiser *et al*[42]	32	27	Adults	Unique score	0	S100A12		-	-	-	-	0.44	< 0.01
	32					S100A12	0.8	81	100	-	-	0.451	0.01
		27				S100A12	0.8	91	100	-	-	0.44	< 0.025

CD: Crohn's disease; UC: Ulcerative colitis; PPV: Positive predictive value; NPV: Negative predictive value.

may contribute to an improved understanding of the relationship between faecal biomarkers and mucosal inflammation and healing.

Firstly, treatment targets in IBD need to be defined and validated. This issue is much broader than the field of faecal biomarkers, but is a clinical and philosophical problem that needs to be urgently resolved. Once resolved, then studies can be performed using established and meaningful endoscopic or other endpoints against which faecal biomarkers can be measured. This includes the assessment of biomarkers against endoscopic and histologic indices, unless there appears to be lack of a validated grading system in IBD for the latter.

Secondly, studies should report their data in clinically meaningful ways including sensitivity, specificity, positive and negative predictive values and accuracy. This will allow comparison between the performances of individual biomarkers and may demonstrate specific advantages of one biomarker over another.

Thirdly, consideration should be made to combining non-endoscopic data to provide the best measure of mucosal inflammation. This could include combinations of clinical symptoms, serum and faecal biomarkers and is likely to be superior to one single parameter. Such analyses will require well-powered studies to enable appropriate analyses.

Finally, the cost-effectiveness of biomarker-driven treatment algorithms needs to be compared with symptoms and endoscopy driven approaches. While biomarker assays are cheaper than endoscopy, the assay costs are still not inconsequential and cost effectiveness must be measured in future studies. These costs should include both direct and indirect costs (the latter are often missed in such analyses and the effect of absenteeism for clinical investigations for patients and their carers should be captured).

In conclusion, Surrogate markers for endoscopic severity in IBD patients are needed for many reasons. Mucosal healing is an important and meaningful objective in the management of this incurable disease. At present, faecal markers seem promising as tools to reflect mucosal healing in IBD, however further research is needed to elucidate their definitive role(s). The variability of study design and endpoints described in this review make it difficult to recommend the routine use of faecal biomarkers in all patients. Nor can one biomarker be suggested to be superior to another given the lack of robust comparative studies. Future research should focus on large studies with clinically meaningful endpoints.

COMMENTS

Background

With regard to disease severity in Crohn's disease and ulcerative colitis patients, several endoscopic, clinical and histologic grading systems are being used. The gold standard for assessing mucosal inflammation and response to therapy in inflammatory bowel disease (IBD) is endoscopy.

Research frontiers

Emerging data suggest that mucosal healing (MH) may be a more reliable target for treatment than clinical and biochemical assessment. MH is associated with improved outcomes in clinical trials. Faecal markers have shown to have multiple advantages in assessing MH and have been suggested as the gold standard for remission.

Innovations and breakthroughs

In addition to MH, the concept of deep remission (encompassing both clinical remission and MH) has been developed, although is yet to be formally tested in clinical trials. Recently a working group of the International Organisation for the study of IBD published a detailed description of potential targets for the management of IBD. The process leading to a "treat to target" approach in IBD has mirrored that seen in other diseases where tight disease control has led to improved patient outcomes.

Applications

Current available studies show a mixed picture of the utility of faecal biomarkers due to a wide variability in study design and endpoints. According to these data, the authors cannot argue for the use or certain cut off values of these markers. If a true treat-to-target approach is to be adopted, accurate and validated data are needed in order to be able to recommend sensitive and specific biomarkers.

Peer-review

In the review, the authors aimed to review the available studies about fecal markers of mucosal healing in IBD. The manuscript is of great clinical importance.

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META-ANALYSIS

Diagnostic accuracy of fluorine-18-fluorodeoxyglucose positron emission tomography in gallbladder cancer: A meta-analysis

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Abstract

AIM: To meta-analyze published data about the diagnostic accuracy of fluorine-18-fluorodeoxyglucose (^{18}F-FDG) positron emission tomography (PET) and PET/computed tomography (PET/CT) in the evaluation of primary tumor in patients with gallbladder cancer (GBCa).

METHODS: A comprehensive literature search of studies published through 30th June 2014 regarding the role of ^{18}F-FDG PET and PET/CT in the evaluation of primary gallbladder cancer (GBCa) was performed. All retrieved studies were reviewed. Pooled sensitivity and specificity of ^{18}F-FDG PET or PET/CT in the evaluation of primary GBCa were calculated. The area under the summary receiving operator characteristics curve (AUC) was calculated to measure the accuracy of these methods. Sub-analyses considering the device used (PET *vs* PET/CT) were carried out.

RESULTS: Twenty-one studies comprising 495 patients who underwent ^{18}F-FDG PET or PET/CT for suspicious GBCa were selected for the systematic review. The meta-analysis of 13 selected studies provided the following results: sensitivity 87% (95%CI: 82%-92%), specificity 78% (95%CI: 68%-86%). The AUC was 0.88. Improvement of sensitivity and specificity was observed when PET/CT was used.

CONCLUSION: ^{18}F-FDG-PET and PET/CT demonstrated to be useful diagnostic imaging methods in the assessment of primary tumor in GBCa patients, nevertheless possible sources of false-negative and false-positive results should be kept in mind. PET/CT seems to have a better diagnostic accuracy than PET alone in this setting.

Key words: Positron emission tomography; Positron emission tomography/computed tomography; Fluorine-18-fluorodeoxyglucose; Gallbladder cancer

Core tip: Fluorine-18-fluorodeoxyglucose-positron emission tomography (PET) and PET/computed tomography (CT) demonstrated to be useful diagnostic imaging methods in the assessment of primary tumor in gallbladder carcinoma patients, nevertheless possible sources of false-negative and false-positive results should be kept in mind. PET/CT seems to have a better diagnostic accuracy than PET alone in this setting.

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INTRODUCTION

Gallbladder carcinoma (GBCa) is the most common carcinoma derived from biliary cells. It is one of the most common carcinoma of gastro-enteric system[1].

The only curative treatment is surgery, but the anatomical complexity of the porto-hepatic system, the morbidity and mortality of liver resection and the risk of tumoral spread induced by the manipulation of unknown GBCa, as well as the absence of effective chemotherapy, explain the high mortality resulting from these tumors[1].

Consequently, accurate evaluation and staging are critical to provide indication to surgery and to avoid unnecessary surgical interventions[2].

Several diagnostic tools have been used in this setting, including ultrasonography (US), computed tomography (CT), magnetic resonance (MR), endoscopic retrograde cholangiopancreatography (ERCP) and percutaneous transhepatic cholangiography (PTC).

Fluorine-18-fluorodeoxyglucose (^{18}F-FDG) positron emission tomography (PET) and PET/CT have been proposed as non-invasive imaging methods to assess the disease extent in cancer patients. Since ^{18}F-FDG is a glucose analogue, this radiopharmaceutical may be very useful in detecting malignant lesions which usually present high glucose metabolism[3]. Hybrid PET/CT device allows enhanced detection and characterization of neoplastic lesions, by combining the functional data obtained by PET with morphological data obtained by CT[3].

Several studies have evaluated the diagnostic accuracy of ^{18}F-FDG-PET or PET/CT in the evaluation of primary tumor in patients with GBCa, reporting different values of sensitivity and specificity[1,2,4-22]. The purpose of our study is to systematically review and meta-analyze published data on this setting in order to provide more evidence-based data.

MATERIALS AND METHODS

Search strategy

A comprehensive computer literature search of PubMed/MEDLINE and EMBASE databases was carried out to find relevant published articles concerning the evaluation of primary tumor in patients with GBCa.

We used a search algorithm based on a combination of the terms: (1) "PET" OR "positron emission tomography"; and (2) "gallbladder" or "gall" or " gall-bladder". Only articles in English language were considered. The search was performed from inception to June 30th, 2014. To expand our search, references of the retrieved articles were also screened for additional studies.

Study selection

Studies or subsets in studies investigating the accuracy of ^{18}F-FDG PET or PET/CT in the evaluation of primary GBCa were eligible for inclusion. Case reports, small case series, review articles, letters, editorials, and conference proceedings were excluded. The following inclusion criteria were applied to select studies for this meta-analysis: (1) original studies in which ^{18}F-FDG PET or PET/CT were performed in patients with GBCa or suspicious GBCa; (2) a sample size of at least nine patients with GBCa or suspicious GBCa; (3) sufficient data to reassess sensitivity and specificity of ^{18}F-FDG PET or PET/CT in detecting the primary tumor in patients with GBCa; and (4) no data overlap.

Three researchers (SA, DAP and CC) independently reviewed titles and abstracts of the retrieved articles, applying the above-mentioned selection criteria. Articles were rejected if clearly ineligible. The same three researchers then independently evaluated the full-text version of the included articles to determine their eligibility for inclusion.

Data extraction

Information about basic study (authors, year of publication, country of origin), study design (prospective or retrospective), patients' characteristics

(number of patients with biliary ducts lesions performing ^{18}F-FDG-PET or PET/CT, mean age, gender) and technical aspects (injected activity of ^{18}F-FDG, time between injection and image acquisition) were collected.

Each study was analyzed to retrieve the number of true-positive (TP), true-negative (TN), false-positive (FP), and false-negative (FN) findings of ^{18}F-FDG PET or PET/CT in patients with GBCa or suspicious GBCa, according to the reference standard. Only studies providing such complete information were finally included in the meta-analysis.

Quality assessment

The 2011 Oxford Center for Evidence-Based Medicine checklist for diagnostic studies was used for quality assessment of the studies included in the meta-analysis. This checklist has 5 major parts as follows: representative spectrum of the patients, consecutive patient recruitment, ascertainment of the gold standard regardless of the index test results, independent blind comparison between the gold standard and index test results, enough explanation of the test to permit replication.

Statistical analysis

Sensitivity and specificity of ^{18}F-FDG PET and PET/CT in the evaluation primary GBCa were obtained from the individual studies, on a per patient-based analysis. We considered as positive a biliary ducts lesion with increased uptake of ^{18}F-FDG, according to the criteria reported by the different authors. When a positive lesion was histologically confirmed as malignant, this was considered a TP lesion, whereas an histologically confirmed benign lesion was considered as a FP lesion. We considered as negative a lesion with no uptake of ^{18}F-FDG: when the lesion was histologically confirmed as malignant, this was considered a FN lesion, whereas a histologically confirmed benign lesion was considered as a TN lesion.

Sensitivity was determined according to the following formula: TP/(TP+FN); specificity was determined according to this formula: TN/(TN+FP). Statistical pooling of the data was performed by means of a random effects model. Pooled data are presented with 95% confidence intervals (95%CI). Heterogeneity between studies was assessed by a I^2 index. A summary receiving operator characteristics (ROC) curve was obtained for selected studies and area under the curve (AUC) was calculated to assess the overall accuracy of ^{18}F-FDG PET and PET/CT.

Subsequently, subgroup analyses were also performed, calculating the pooled sensitivity and specificity of ^{18}F-FDG PET and PET/CT in two groups based on the different device used (PET or PET/CT).

Statistical analyses were performed using MetaDiSc statistical software version 1.4.

RESULTS

Literature search

The comprehensive computer literature search from PubMed/MEDLINE and EMBASE databases revealed 250 articles. Reviewing titles and abstracts, 229 records were excluded as reviews, editorials or letters, case reports or case series or no direct link with the main subject. Finally, 21 articles including 495 patients were selected and were eligible for the systematic review[1,2,4-22]; no additional studies were found screening the references of these articles. The characteristics of the included studies are presented in Table 1, Table 2 and Table 3.

Qualitative analysis (systematic review)

Using the database search, 21 original articles written over the past 11 years were selected[1,2,4-22]. About the study design, 4 of these studies were prospective[1,7,10,15], 12 retrospective[2,8,9,12-14,16,18-22] and in 5 articles this information was not provided[4-6,11,17]. Ten studies used hybrid PET/CT[1,2,10,11,15,16,18,19,21,22], ten studies used PET only[4-9,12-14,20], one study used both PET or PET/CT[17]. Heterogeneous technical aspects between the included studies were found (Table 2). PET image analysis was performed by using qualitative criteria (visual analysis) in all the included studies[1,2,4-22] and adjunctive semi-quantitative criteria [based on the calculation of the standardized uptake value (SUV)] in 15 articles[1,2,6,8-10,12,14-17,19-22].

The reference standard used to validate the ^{18}F-FDG PET or PET/CT findings in the included studies were quite different.

Quantitative analysis (meta-analysis)

Only 13 over 21 studies included in the systematic review had sufficient data to calculate the pooled sensitivity[1,2,4-6,8-10,12,13,16,18,20], whereas only 9 studies[1,4-9,12,13,18] provided information about TN and FP lesions, thus allowing to assess pooled specificity. The diagnostic accuracy values of ^{18}F-FDG PET and PET/CT in the studies included in the meta-analysis are presented in Figures 1 and 2.

Sensitivity and specificity values of ^{18}F-FDG PET or PET/CT on a per patient-based analysis ranged from 69% to 100% and from 44% to 100%, with pooled estimates of 87% (95%CI: 82%-92%) and 78% (95%CI: 68%-86%), respectively. The area under the summary ROC curve was 0.88 (Figure 3). The included studies showed mild statistical heterogeneity (I^2: 42%) in their estimate of sensitivity only.

Subgroup analyses considering the different device used (PET or PET/CT) were performed. In studies in which ^{18}F-FDG PET was used, values of sensitivity (8 eligible studies) and specificity (8 eligible studies) on a per patient-based analysis ranged from 69% to 100% and from 0% to 100%, respectively, with

Table 1 Basic characteristics of the studies included in the systematic review

Ref.	Year	Country	Study design	Patients performing ^{18}F-FDG-PET or PET/CT with GB lesions	Mean age (yr)	Gender (%male)
Koh *et al*[4]	2003	Japan	NR	16	68	44%
Anderson *et al*[5]	2004	United States	NR	14	65	57%
Rodríguez-Fernández *et al*[6]	2004	Spain	NR	16	68	31%
Wakabayashi *et al*[7]	2005	Japan	Prospective	30	71	50%
Nishiyama *et al*[8]	2006	Japan	Retrospective	32	70	37%
Oe *et al*[9]	2006	Japan	Retrospective	12	68	66%
Petrowsky *et al*[10]	2006	Switzerland	Prospective	14	NR	NR
Shukla *et al*[11]	2008	India	NR	24	45	33%
Corvera *et al*[12]	2008	United States	Retrospective	41	62	52%
Furukawa *et al*[13]	2008	Japan	Retrospective	18	NR	NR
Furukawa *et al*[14]	2009	Japan	Retrospective	18	NR	NR
Butte *et al*[15]	2009	Chile	Prospective	53	57	20%
Lee *et al*[16]	2010	South Korea	Retrospective	16	NR	NR
Zhu *et al*[17]	2010	United States	NR	10	NR	NR
Lee *et al*[18]	2012	South Korea	Retrospective	20	65	45%
Kumar *et al*[19]	2012	India	Retrospective	49	52	31%
Yamada *et al*[20]	2012	Japan	Retrospective	14	NR	NR
Albazaz *et al*[2]	2013	United Kingdom	Retrospective	30	NR	NR
Lee *et al*[21]	2013	South Korea	Retrospective	9	NR	NR
Onal *et al*[22]	2013	Turkey	Retrospective	10	63	70%
Ramos-Font *et al*[1]	2014	Spain	Prospective	49	68	43%

^{18}F-FDG: Fluorine-18-fluorodeoxyglucose; PET: Positron emission tomography; CT: Computed tomography; NR: Not reported; GB: Gallbladder; GBCa: Gallbladder carcinoma.

Table 2 Technical characteristics of the studies included in the systematic review

Ref.	Year	Device	^{18}F-FDG mean injected dose (MBq)	Time between ^{18}F-FDG injection and image acquisition (min)	Image analysis	Other imaging methods performed
Koh *et al*[4]	2003	PET	185	60	Visual	CT
Anderson *et al*[5]	2004	PET	370	60	Visual	CT, MRI
Rodríguez-Fernández *et al*[6]	2004	PET	370	45-60	Visual and semiquantitative	US, CT
Wakabayashi *et al*[7]	2005	PET	185	60	Visual	CT
Nishiyama *et al*[8]	2006	PET	185-370	40-55	Visual and semiquantitative	US, CT
Oe *et al*[9]	2006	PET	3/kg	62-146	Visual and semiquantitative	US, CT, MRI
Petrowsky *et al*[10]	2006	PET/CT	370	45	Visual and semiquantitative	CT
Shukla *et al*[11]	2008	PET/CT	370	60	Visual	CT
Corvera *et al*[12]	2008	PET	370-555	NR	Visual and semiquantitative	CT, MRI
Furukawa *et al*[13]	2008	PET	200-250	60	Visual	CT
Furukawa *et al*[14]	2009	PET	200-250	60	Visual and Semiquantitative	CT, MRI, PTC
Butte *et al*[15]	2009	PET/CT	370	60	Visual and semiquantitative	NR
Lee *et al*[16]	2010	PET/CT	370-555	60	Visual and semiquantitative	CT
Zhu *et al*[17]	2010	PET or PET/TC	370-555	45	Visual and Semiquantitative	CT
Lee *et al*[18]	2012	PET/CT	370	45-60	Visual	NR
Kumar *et al*[19]	2012	PET/CT	5.5/kg	60	Visual and semiquantitative	NR
Yamada *et al*[20]	2012	PET	4.5/kg	60	Visual and semiquantitative	NR
Albazaz *et al*[2]	2013	PET/CT	400	60	Visual and semiquantitative	CT, MRI
Lee *et al*[21]	2013	PET/CT	370-555	60	Visual and semiquantitative	CT, MRCP, ERCP, EUS
Onal *et al*[22]	2013	PET/CT	370-555	60	Visual and Semiquantitative	MRI, CT
Ramos-Font *et al*[1]	2014	PET/CT	370	60	Visual and semiquantitative	NR

^{18}F-FDG: Fluorine-18-fluorodeoxyglucose; PET: Positron emission tomography; CT: Computed tomography; MR: Magnetic resonance; US: Ultrasonography; CR: Chest radiography; PTC: Percutaneous transhepatic cholangiography; NR: Not reported; CT: Computed tomography; ERCP: Endoscopic retrograde cholangiopancreatography.

pooled estimates of 83% (95%CI: 75%-90%) and 71% (95%CI: 55%-84%), respectively. No statistical heterogeneity was found in these sub-analyses.

In studies in which hybrid ^{18}F-FDG PET/CT was used, values of sensitivity (5 eligible studies) and specificity (4 eligible studies) on a per patient-based analysis ranged from 80% to 100% and from 0% to 88%, respectively, with pooled estimates of 93% (95%CI: 85%-97%) and 80% (95%CI: 66%-90%), respectively. Statistical heterogeneity was found both in their estimate of sensitivity (I^2 = 65%) and specificity (I^2 = 59%).

Table 3 Diagnostic accuracy data of fluorine-18-fluorodeoxyglucose-positron emission tomography and positron emission tomography/computed tomography on a per patient-based analysis

Ref.	Year	Overall				PET				PET/CT			
		TP	FP	FN	TN	TP	FP	FN	TN	TP	FP	FN	TN
Koh *et al*[4]	2003	6	1	2	7	6	1	2	7	NR	NR	NR	NR
Anderson *et al*[5]	2004	7	1	2	4	7	1	2	4	NR	NR	NR	NR
Rodríguez-Fernández *et al*[6]	2004	4	2	1	9	4	2	1	9	NR	NR	NR	NR
Nishiyama *et al*[8]	2006	19	5	4	4	19	5	4	4	NR	NR	NR	NR
Oe *et al*[9]	2006	3	1	0	2	3	1	0	2	NR	NR	NR	NR
Petrowsky *et al*[10]	2006	14	1	0	0	NR	NR	NR	NR	14	1	0	0
Corvera *et al*[12]	2008	24	1	4	2	24	1	4	2	NR	NR	NR	NR
Furukawa *et al*[13]	2008	17	0	1	2	17	0	1	0	NR	NR	NR	NR
Lee *et al*[16]	2010	14	0	2	0	NR	NR	NR	NR	14	0	2	0
Lee *et al*[18]	2012	16	6	4	24	NR	NR	NR	NR	16	6	4	24
Yamada *et al*[20]	2012	9	1	4	0	9	1	4	0	NR	NR	NR	NR
Albazaz *et al*[2]	2013	15	1	0	0	NR	NR	NR	NR	15	1	0	0
Ramos-Font *et al*[1]	2014	20	2	0	15	NR	NR	NR	NR	25	2	0	22

NR: Not reported; IH-CCA: Intrahepatic cholangiocarcinoma; H-CCA: Hilar cholangiocarcinoma; EH-CCA: Extrahepatic cholangiocarcinoma; TP: True positive; FP: False positive; FN: False negative; TN: True negative.

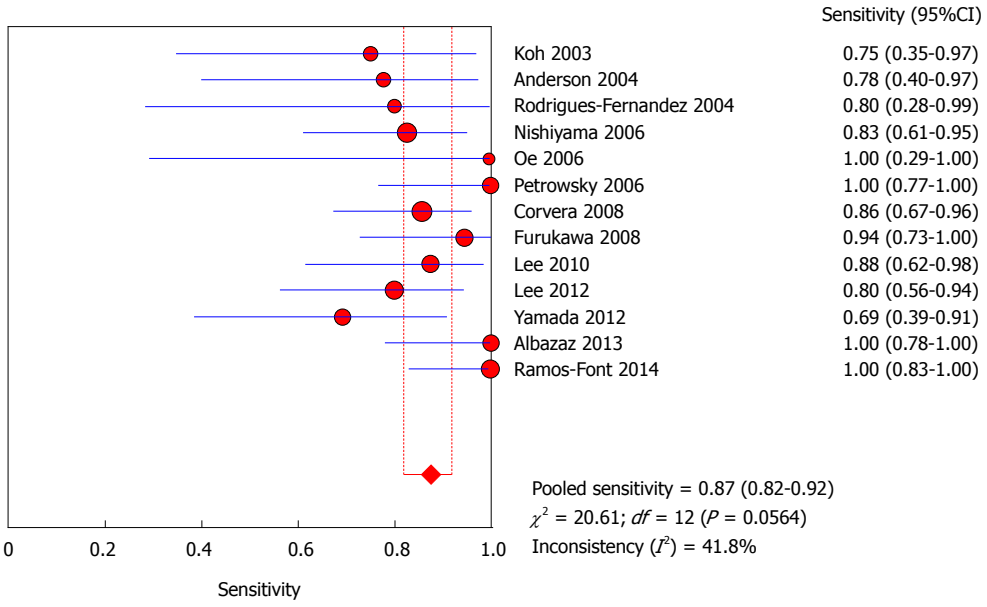

Figure 1 Sensitivity values of fluorine-18-fluorodeoxyglucose positron emission tomography and positron emission tomography/computed tomography in the studies (*n* = 13) included in the meta-analysis.

DISCUSSION

To the best of our knowledge, this meta-analysis is the first to evaluate the diagnostic accuracy of ^{18}F-FDG PET and PET/CT in the evaluation of primary tumor in patients with GBCa. Several studies have used ^{18}F-FDG PET or PET/CT in this setting reporting different values of sensitivity and specificity. However, many of these studies have limited power, analyzing only relatively small numbers of patients. In order to derive more robust estimates of the diagnostic accuracy of ^{18}F-FDG PET or PET/CT in this setting we pooled published studies. A systematic review process was adopted in ascertaining studies, thereby avoiding selection bias[23].

Pooled results of our meta-analysis indicate that ^{18}F-FDG PET or PET/CT have a good sensitivity (87%) and specificity (78%) in the evaluation of primary tumor in patients with GBCa. Furthermore, the value of the AUC (0.88) demonstrates that ^{18}F-FDG PET or PET/CT are accurate diagnostic methods in this setting.

Possible sources of false-positive results (such as inflammatory diseases of the gallbladder) and false negative results (such as small size and/or low-grade tumors) should be considered.

A subgroup analysis considering different device used (PET *vs* PET/CT) was performed. We found higher pooled sensitivity and specificity when PET/CT was used compared to PET. This is not surprising

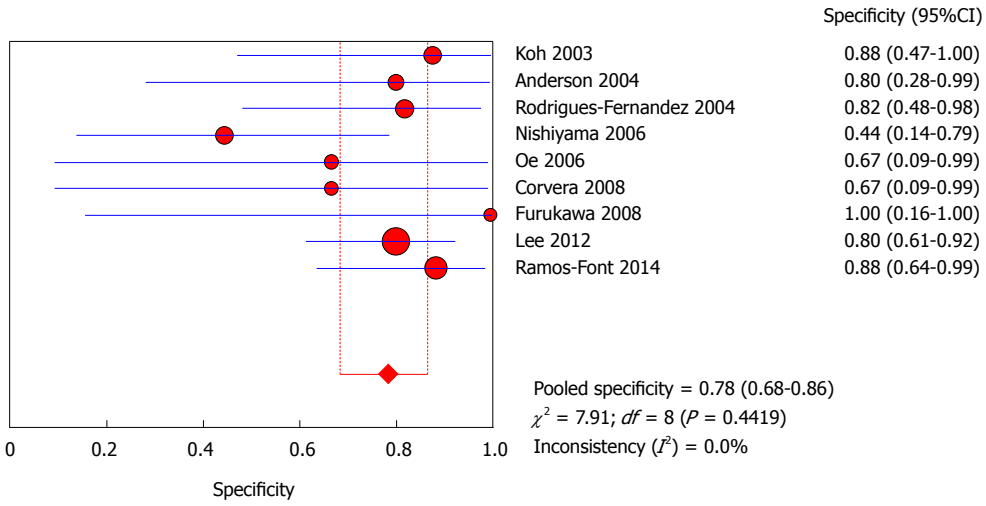

Figure 2 Specificity values of fluorine-18-fluorodeoxyglucose positron emission tomography and positron emission tomography/computed tomography in the studies (*n* = 9) included in the meta-analysis.

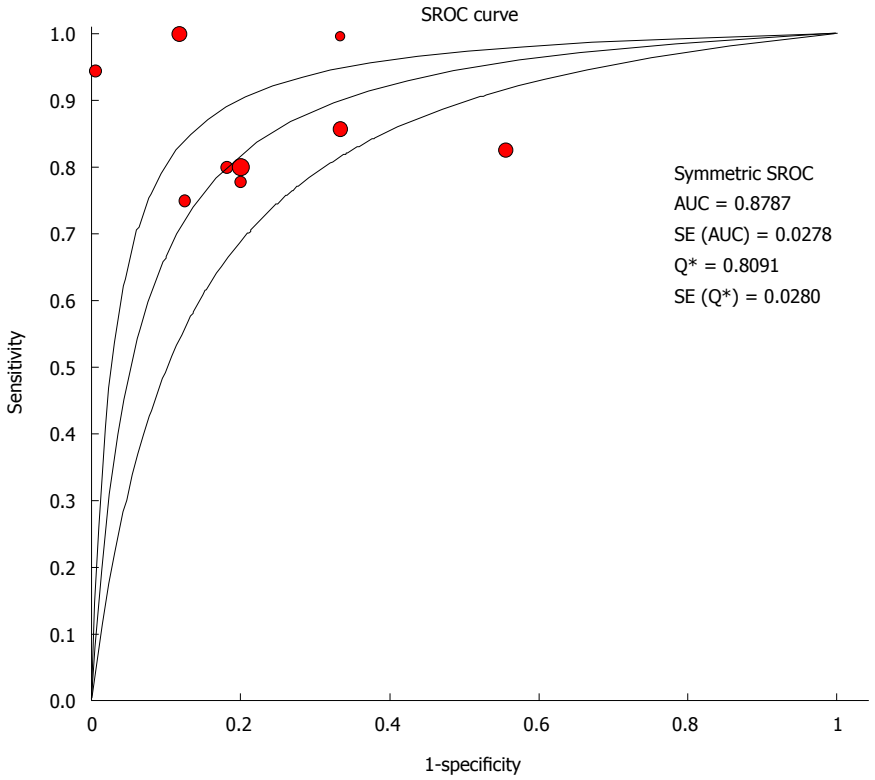

Figure 3 Receiving operator characteristics curve analysis of accuracy values of studies (*n* = 9) included in the meta-analysis.

considering the higher diagnostic accuracy of PET/CT compared to PET imaging[3].

Regarding the diagnostic work-up of patients with GBCa, ^{18}F-FDG PET and PET/CT may have little diagnostic advantage over traditional imaging modalities in detecting primary GBCa[2]. ^{18}F-FDG PET and PET/CT can be complementary to US, MR, CT, PTC and ERCP in staging GBCa patients. Since ^{18}F-FDG PET is a whole-body scanning technique, it allows detection of unsuspected metastatic lymph nodes or distant spread that may lead to major changes in the surgical management of patients with biliary tract cancer[20]. Nevertheless, the diagnostic performance of ^{18}F-FDG PET or PET/CT in detecting metastatic lymph nodes or distant spread was not object of our analysis.

This meta-analysis has some limitations such as the heterogeneity between the studies, the publication bias and the limited number of articles available for the subgroup analysis.

Heterogeneity between studies may represent a potential source of bias in a meta-analysis. This heterogeneity is likely to arise through diversity in

methodological aspects between different studies. The baseline differences among the patients in the included studies, the reference standard used, and the study quality may contribute to the heterogeneity of the results too. In our pooled analysis the included studies were statistically mild heterogeneous in their estimate of pooled sensitivity only.

Publication bias is a major concern in all meta-analyses as studies reporting significant findings are more likely to be published than those reporting non-significant results. Indeed, it is not unusual for small-sized early studies to report a positive relationship that subsequent larger studies fail to replicate. We cannot exclude a publication bias in our meta-analysis.

Only a limited number of articles were available for the subgroup analysis based on the different device used (PET *vs* PET/CT) and this could limit the statistical power of the subgroup analysis.

Overall, ^{18}F-FDG PET and PET/CT demonstrated to be quite accurate non-invasive tools in the evaluation of primary tumors in patients with GBCa. Nevertheless, multicentric studies and cost-effectiveness analyses about the role of ^{18}F-FDG PET/CT in this setting are needed.

^{18}F-FDG-PET and PET/CT demonstrated to be quite accurate diagnostic imaging methods in the evaluation of primary tumors in patients with GBCa. PET/CT seems to have a better diagnostic accuracy than PET alone in this setting.

COMMENTS

Background

Several studies have evaluated the diagnostic accuracy of fluorine-18-fluorodeoxyglucose (^{18}F-FDG) positron emission tomography (PET) or PET/computed tomography (CT) in the evaluation of primary tumor in patients with gallbladder cancer (GBCa), reporting different values of sensitivity and specificity.

Applications

^{18}F-FDG-PET and PET/CT demonstrated to be quite accurate diagnostic imaging methods in the evaluation of primary tumors in patients with GBCa. PET/CT seems to have a better diagnostic accuracy than PET alone in this setting.

Peer-review

It is an interesting article. This article was well designed meta-analysis about gallbladder cancer diagnosis.

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