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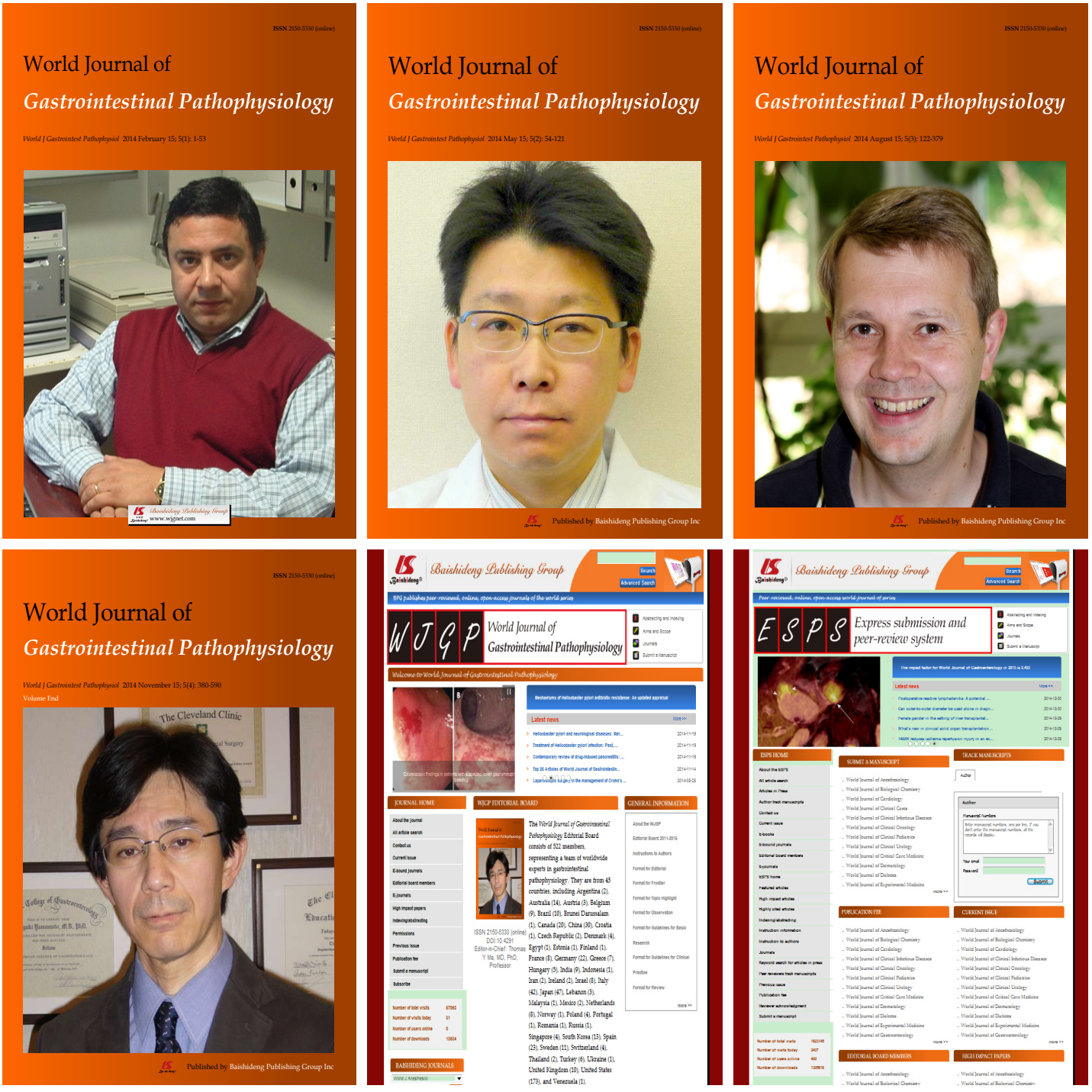

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World Journal of Gastrointestinal Pathophysiology (*World J Gastrointest Pathophysiol*, *WJGP*, online ISSN 2150-5330, DOI: 10.4291), is a peer-reviewed open access academic journal that aims to guide clinical practice and improve diagnostic and therapeutic skills of clinicians.

WJGP is to report rapidly the most recent results in basic and clinical research on gastrointestinal pathophysiology, including all aspects of normal or abnormal function of the gastrointestinal tract, hepatobiliary system, and pancreas. *WJGP* specifically covers growth and development, digestion, secretion, absorption, metabolism and motility relative to the gastrointestinal organs, as well as immune and inflammatory processes, and neural, endocrine and circulatory control mechanisms that affect these organs. This journal will also report new methods and techniques in gastrointestinal pathophysiological research.

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TOPIC HIGHLIGHT

WJGP 5th Anniversary Special Issues (3): Pancreatitis

Prevention of post-ERCP pancreatitis

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Abstract

Post-procedure pancreatitis is the most common complication of endoscopic retrograde cholangio pancreatography (ERCP) and carries a high morbidity and mortality occurring in at least 3%-5% of all procedures. We reviewed the available literature searching for "ERCP" and "pancreatitis" and "post-ERCP pancreatitis". in PubMed and Medline. This review looks at the diagnosis, risk factors, causes and methods of preventing post-procedure pancreatitis. These include the evidence for patient selection, endoscopic techniques and pharmacological prophylaxis of ERCP induced pancreatitis. Selecting the right patient for the procedure by a risk benefits assessment is the best way of avoiding unnecessary ERCPs. Risk is particularly high in young women with sphincter of Oddi dysfunction (SOD). Many of the trials reviewed have rather few numbers of subjects and hence difficult to appraise. Meta-analyses have helped screen for promising modalities of prophylaxis. At present, evidence is emerging that pancreatic stenting of patients with SOD and rectally administered non-steroidal anti-inflammatory drugs in a large unselected trial reduce the risk of post-procedure pancreatitis. A recent meta-analysis have demonstrated that rectally administered indomethecin, just before or after ERCP is associated with significantly lower rate of pancreatitis compared with placebo [OR = 0.49 (0.34-0.71); *P* = 0.0002]. Number needed to treat was 20. It is likely that one of these prophylactic measures will begin to be increasingly practised in high risk groups.

Key words: Acute pancreatitis; Endoscopic retrograde cholangio pancreatography

Core tip: Select patients carefully, and give high risk patients rectal indomethacin.

Wong LL, Tsai HH. Prevention of post-ERCP pancreatitis. *World J Gastrointest Pathophysiol* 2014; 5(1): 1-10 Available from: URL: http://www.wjgnet.com/2150-5330/full/v5/i1/1.htm DOI: http://dx.doi.org/10.4291/wjgp.v5.i1.1

INTRODUCTION

Pancreatitis is the most common complication of endoscopic retrograde cholangio pancreatography (ERCP) and carries a high morbidity and mortality[1,2]. There is a 3%-5% incidence of this complication occurring, as shown in various large clinical studies[2-4]. A systematic survey of 21 studies involving 16855 patients (1987-2003) found a 3.5% occurrence of post-ERCP pancreatitis. 0.4% of patients had severe pancreatitis with 0.11% deaths[5].

Predicting pancreatitis after ERCP can be very difficult but there have been numerous studies that have identified factors that increase the risk for post-ERCP pancreatitis. These can have a cumulative effect when multiple factors are present. There are multiple procedures and pharmacological interventions that have been studied to prevent post-ERCP pancreatitis. This article describes these some of these interventions and includes the latest studies.

Table 1 Consensus definition of post-endoscopic retrograde cholangio pancreatography pancreatitis

Severity of pancreatitis	Definition
Mild	Clinical pancreatitis, amylase at least 3 × normal > 24 h after procedure, requiring unplanned admission or prolongation of planned admission to 2-3 d
Moderate	Hospitalisation of 4-10 d
Severe	Hospitalisation of > 10 d, haemorrhagic pancreatitis, pancreatic necrosis or pseudocyst, or need for intervention (percutaneous drainage or surgery)

DIAGNOSIS OF POST-ERCP PANCREATITIS

Post-ERCP pancreatitis is defined as acute pancreatitis occurring following an ERCP procedure. This consists of the development of new pancreatic-type abdominal pain associated with hyperamylasemia of three times the upper-limit of normal, occurring 24 h after an ERCP requiring hospital admission. Severity of post-ERCP pancreatitis is graded based on length of hospital admission and need for intervention. It can be divided into mild, moderate and severe (Table 1), based on a consensus definition[6].

Freeman *et al*[1] studied the complication rate that occurred in 2347 patients undergoing endoscopic biliary sphincterotomy. Acute pancreatitis occurred in 127 patients (5.4%). Mild post-ERCP pancreatitis occurred in 53 (2.3%), moderate in 65 (2.8%) and severe in 9 (0.4%). Of the latter, one died of retroperitoneal perforation, one required percutaneous drainage of a pseudocyst and three required surgical drainage.

RISK FACTORS FOR POST-ERCP PANCREATITIS

Many studies have looked into factors that increase the risk of post-ERCP pancreatitis. These can be divided into patient-related risk factors, endoscopist-related risk factors and procedure-related risk factors. Table 2 summarises the general consensus of risk factors for post-ERCP pancreatitis[3,7,8]. These factors should alert the endoscopist to take special precautions in preventing post-ERCP pancreatitis[9]. In addition, there is a cumulative effect for patients with multiple risk factors. For example, a young woman with suspected sphincter of Oddi dysfunction, normal bilirubin, difficult cannulation and absence of bile duct stones has an associated increased risk of pancreatitis of 40%[10,11].

There are other factors that have been identified which require further studies. One retrospective study identified taking pancreato-toxic drugs (oestrogen, azathioprine, valproic acid, mesalazine, morphine derivatives and prednisone) increased the occurrence of post-ERCP pancreatitis (OR = 3.7)[12].

Another retrospective study of 506 patients identified angiotensin receptor blockers and smoking as independent risk factors for post-ERCP pancreatitis[13] whereas a recent case-control study of 6505 patients identified smoking and chronic liver disease as factors that reduced the risk of post-ERCP pancreatitis[14].

Table 2 Risk factors for post-endoscopic retrograde cholangio pancreatography pancreatitis

Risk factors for post ERCP pancreatitis	
Patient-related factors	Younger age
	Female sex
	Normal serum bilirubin
	Recurrent pancreatitis
	Prior ERCP-induced pancreatitis
	Sphincter of Oddi dysfunction
Endoscopist-related factors	Difficult cannulation
	Pancreatic duct injection
	Sphincter of Oddi manometry
	Precut sphincterotomy
	Pancreatic sphincterotomy
	Minor papilla sphincterotomy
Procedure-related factors	Trainee involvement in procedure

ERCP: Endoscopic retrograde cholangio pancreatography.

Testoni *et al*[7] conducted a large prospective multicentre trial (total of 3635 ERCP procedures) and showed that the rate of post-ERCP pancreatitis did not differ between high- and low-volume centres (3.9% *vs* 3.1%). However, the high-volume centres treated a larger proportion of patients at high-risk of pancreatitis and did a significantly greater number of difficult procedures. In another large multicentre prospective trial (2347 patients), case volume did not affect incidence of pancreatitis although the multivariate model indicated low case volume was independently associated with higher overall rate of complications[1].

Operator experience has been difficult to demonstrate as a risk factor for post-ERCP pancreatitis due to the heterogeneity of studies with variable case volume and case mix. One French study showed no risk associated with operator inexperience[14].

In the multivariate analysis of a randomised controlled multicentre study by Cheng *et al*[8], trainee involvement in the procedure was found to be a risk factor (OR = 1.5) for development of post-ERCP pancreatitis.

Biliary stenting was found to be an independent risk factor for pancreatitis in a single-centre prospective study by Wilcox *et al*[15]. The commonest indication for stent placement was pancreaticobiliary malignancy (37% of patients). Another retrospective study on patients undergoing ERCP for malignant biliary obstruction found the frequency of post-ERCP pancreatitis was significantly higher with placement of self-expanding metal stents compared with a plastic stent[16].

MECHANISM OF POST-ERCP PANCREATITIS

There are various mechanisms proposed in the pathogenesis of post-ERCP pancreatitis[17,18]. These include: (1)

mechanical injury from instrumentation of papilla and pancreatic duct; (2) thermal injury following application of electrosurgical current during biliary or pancreatic sphincterotomy; (3) hydrostatic injury - following injection of contrast medium into the pancreatic duct of from infusion of water or saline solution during sphincter manometry; (4) chemical or allergic injury following injection of contrast medium into the pancreatic duct; (5) enzymatic injury with intraluminal activation of proteolytic enzymes; and (6) infection from contaminated endoscope and accessories.

Preventive measures are aimed at interrupting the cascade of events resulting in the premature activation of proteolytic enzymes, autodigestion and impaired acinar secretion with subsequent clinical manifestations of local and systemic effects of pancreatitis[17].

PREVENTION OF POST-ERCP PANCREATITIS

ERCP technique

Cannulation: Various methods to ease cannulation of the bile duct and reduce trauma have been studied with view of reducing the risk of post-ERCP pancreatitis.

In general, guidewire technique to facilitate bile duct cannulation has been shown to improve primary biliary duct cannulation but incidence of post-ERCP pancreatitis has not been consistently shown to be reduced by this technique.

In a meta-analysis of five randomised controlled trials (RCTs), guidewire cannulation was shown to lower post-ERCP pancreatitis (rates 0%-3%) compared to standard contrast-injection method (rates 4%-12%) and increase primary cannulation rates compared to the standard method (OR = 2.05)[19].

A Cochrane meta-analysis of 12 RCTs (3450 patients) similarly found that post-ERCP pancreatitis incidence was lower in the wire-guided cannulation (WGC) group (3.5%) compared to contrast-assisted cannulation technique (6.7%) and primary cannulation rates were higher in the WCG group (84% *vs* 77%, RR = 1.07). However, WGC may not prevent post-ERCP pancreatitis in patients with suspected Sphincter-of-Oddi dysfunction and unintentional pancreatic duct guidewire cannulation[20].

In contrast, a recent crossover multicentre randomised controlled trial involving 322 patients compared wire-guided biliary cannulation with conventional cannulation technique - the trial found that the incidence of post-ERCP pancreatitis was similar in both groups (6.1% *vs* 6.3%, $P = 0.95$). Primary biliary cannulation rate was similar for both groups as well (83% *vs* 87%)[21].

Another prospective trial involving 1249 patients did not find any significant difference in the rates of post-ERCP pancreatitis with the guidewire technique compared with sphincterotome and contrast injection method[22].

Many advanced endoscopists use a hybrid of the two techniques (wire probes with minimal contrast to outline distal duct course) which avoid dissections or passage of the guidewire out of a side branch of the pancreatic duct. This hybrid technique however has not been formally evaluated[23].

Electrocautery: Thermal injury following application of electrosurgical current during biliary or pancreatic sphincterotomy is thought to contribute to causing post-ERCP pancreatitis. A number of studies have been conducted to compare pure cut current with blended current and bipolar *vs* monopolar electrocautery. These have produced mixed results. A meta-analysis of four trials (total: 804 patients) comparing pure current to mixed current in patients who underwent sphincterotomy found no significant difference in the rates of pancreatitis. Pure current was however associated with more episodes of bleeding, primarily mild bleeding[24]. The use of sequential combination of pure cut and blended current for sphincterotomy was studied in 142 patients - this did not change the rate of post-ERCP pancreatitis but did cause less visible bleeding than pure cut alone[25].

Pancreatic stenting: Pancreatic duct obstruction or impaired pancreatic drainage from papillary oedema or spasm of the sphincter of Oddi has been postulated to cause post-ERCP pancreatitis[17]. Numerous studies have looked into the prophylactic placement of a pancreatic stent to prevent post-ERCP pancreatitis. Due to their variability in indications for stenting, interventions and outcome measures - comparisons and conclusions can be difficult. A few trials have shown that pancreatic stent insertion reduces the rate and severity of post-ERCP pancreatitis after difficult cannulation, needle-knife precut, biliary sphincterotomy for sphincter of Oddi dysfunction (SOD) and manometry, pancreatic sphincterotomy, endoscopic ampullectomy and endoscopic balloon dilation[26-33].

A recent meta-analysis of randomised controlled trials (RCTs) comparing pancreatic stent placement and the subsequent incidence of post-ERCP pancreatitis enrolled 14 studies (total: 1541 patients). This found that pancreatic stent placement was associated with significant reduction of post-ERCP pancreatitis (RR=0.39, 95%CI: 0.29-0.53, $P < 0.001$) as compared with no stent placement. Subgroup analysis demonstrated that pancreatic stent placement was effective for both high-risk and mixed case groups[34].

Another meta-analysis by Choudhary *et al*[35] analysed eight RCTs (656 patients) and this showed that prophylactic pancreatic stents decreased the odds of post-ERCP pancreatitis (OR = 0.22; 95%CI: 0.12-0.38, $P < 0.01$) with an absolute risk difference of 13%.

Pancreatic stenting comes with some limitations. It is associated with complications such as stent-related ductal injury and strictures[36]. Many endoscopists and assistants are unfamiliar with the placement of pancreatic stents. In addition, unsuccessful stent placement can itself be associated with a risk of pancreatitis. Freeman *et al*[37] conducted a prospective study of 225 high risk ERCPs.

Table 3 Pharmacological agents studied according to postulated mechanism of action

Postulated mechanism of action	Agents
Interruption of inflam matory cascade	NSAIDs, steroids, interleukin-10, allopurinol, adrenaline spray, pentoxifylline, platelet-activating factor-acetylhidrolase, semapimod, aprepitant, risperidone
Reduction of pancreatic enzyme secretion	Octreotide, somatostatin, calcitonin
Inhibition of protease activity	Gabexate mesilate, heparin, ulinastatin, nafamostat, magnesium sulphate
Reduction of Sphincter-of-Oddi pressure	Nitroglycerin, nifedipine, botulinum toxin, lidocaine, secretin, phosphodiesterase inhibitor type 5
Prevention of infection	Antibiotics
Anti-oxidants	Beta-carotene, N-acetylcysteine, sodium selenite
Anti-metabolites	5-fluorouracil

NSAIDs: Non-steroidal anti-inflammatory drugs.

Pancreatitis occurred in two out of three (66.7%) patients in whom stent insertion failed *vs* 32 of 222 (14.4%) patients with successful insertion ($P = 0.06$).

Follow-up evaluation is necessary to ensure passage or removal of stent and placement can be technically difficult. The optimal timing for stent placement and duration for stent to remain in place is unknown. There is also variability in the type of stent used[17,33]. Short 5 French stents are easier to deploy and are more likely to migrate spontaneously compared with long 3 French stents. However, they do not confer a benefit in terms of pancreatitis risk reduction. The optimal duration for stents to remain in place is unknown. Chahal *et al*[38] compared the outcomes of a short straight 5 French stent without an inner flange with an unflanged long single pigtail 3 French stent. They found a significantly higher placement failure rate in the 3 French group (8.3% *vs* 0%, $P = 0.0003$), a higher spontaneous dislodgement rate in the 5 French group (98% *vs* 88% for 3 Fr, $P = 0.0001$) and a non-significant higher pancreatitis rate (14% *vs* 9%, $P = 0.3$).

Pharmacological prophylaxis

Since the introduction of ERCP, numerous studies have been carried out in the pursuit to discover the most effective pharmacological prophylactic agent against post-ERCP pancreatitis. These were done based on the postulated mechanisms of action through which post-ERCP pancreatitis occurred (Table 3)[39,40].

Interruption of inflammatory cascade (anti-inflammatory): Non-steroidal anti-inflammatory drugs: non-steroidal anti-inflammatory drugs (NSAIDs) have been studied for their inhibitory properties on phospholipid A2 (PLA_2) and prostaglandins, which lead to interruption of the inflammatory cascade of acute pancreatitis[41]. A Finnish group, Mäkelä *et al*[42] studied the in-vitro inhibition of PLA_2 in acute pancreatitis by 17 different pharmacological agents. They found that indomethacin was the most potent of the agents in inhibiting PLA_2 activity in the serum from patients with acute pancreatitis followed by diclofenac.

Murray *et al*[43] conducted a prospective, randomised, double-blind controlled trial involving 220 patients. In the twenty-four patients (11%) who developed acute pancreatitis, 7 had received 100mg diclofenac suppository given immediately after ERCP *vs* 17 who received placebo ($P < 0.05$). They concluded that rectal diclofenac given immediately after ERCP can reduce the incidence of acute pancreatitis.

Since then, three meta-analysis have been published, analysing the effect of NSAIDs in preventing post-ERCP pancreatitis. The results of each meta-analyses are as follows: (1) Elmunzer *et al*[44]: Four RCTs (912 patients) evaluating rectal NSAIDs (indomethacin or diclofenac) administration in the peri-procedure period were analysed. This found a significant reduced incidence of pancreatitis with pooled relative risk of 0.36. The pooled number needed to treat with NSAIDs to prevent one episode of pancreatitis was 15; (2) Dai *et al*[45]: Six RCTs (1300 patients) were analysed. These included the 4 RCTs in the above-mentioned meta-analysis as well as two additional trials. Two trials used rectal diclofenac, three used rectal indomethacin and one used oral diclofenac. The risk of pancreatitis was lower in the NSAID group than in the placebo group (OR = 0.46, $P < 0.0001$)[45]; and (3) Ding *et al*[46]: Meta-analysis of ten RCTs (2269 patients) showed that NSAIDs decreased the overall incidence of post-ERCP pancreatitis (RR = 0.57, $P = 0.007$) with an absolute risk reduction of 5.9% and number needed to treat: 17. In addition, NSAIDs use decreased the incidence of moderate to severe post-ERCP pancreatitis (RR = 0.46, $P = 0.002$). This meta-analyses included studies that were heterogenous in NSAIDs-type (indomethacin, diclofenac or valdecoxib) and route of administration. Rectal administration of NSAIDs was associated with a decreased risk of post-ERCP pancreatitis in all six trials that used this route while the other routes studied in 4 studies (oral, intramuscular, intravenous and intraduodenal) were not.

Rectal administration is the most effective route for NSAIDs in post-ERCP prevention. This is postulated to be due to wider bioavailability compared to oral route (with significant first-pass metabolism) and the quicker peak plasma NSAIDs concentrations (30 min for rectal route *vs* 2 h for oral route)[47,48].

All the trials showed no adverse effects from NSAIDs administration to patients. However, limitations to the meta-analyses were differences in pharmacological manipulation (timing, route of administration and choice of drug), inconsistent use of pancreatic stenting, inclusion of both high-risk and low-risk patients and differences in ERCP procedures (*e.g.*, number of cannulations, number of pancreatic duct injections, whether sphincterotomy was performed). In addition, different definitions of pancreatitis were used [some used 4 × upper limit of normal (ULN) hyperamylasemia while some used 3 × ULN with abdominal pain][44,45]

The latest multi-centre trial by Elmunzer *et al*[49] was carried out using a randomised, placebo-controlled and double-blind method. This compared rectal indomethacin *vs* placebo immediately after ERCP. A total of 602 patients were enrolled of which 82% were high-risk (suspected sphincter of Oddi dysfunction). Rectal indomethacin was found to significantly reduce the incidence of post-ERCP pancreatitis (9.2% *vs* 16.9%, $P = 0.005$).

A recent meta-analysis have demonstrated that rectally administered indomethacin, just before or after ERCP is associated with significantly lower rate of pancreatitis compared with placebo [OR = 0.49 (0.34-0.71); $P = 0.0002$]. Number needed to treat was 20. Moreover they found that in subgroup analysis, the difference remained unchanged for average-risk population [OR = 0.49 (0.28-0.85); $P = 0.01$] or in preventing severe PEP [OR = 0.41 (0.21-0.78); $P = 0.007$][50]. The European Society of Gastrointestinal Endoscopy published guidelines in 2010 with grade A recommendation for the administration of rectal diclofenac 100 mg or indomethacin immediately before or after ERCP as post-ERCP prophylaxis[51]. The United States and United Kingdom however have not yet come to a consensus regarding this.

The available evidence suggests that prophylactic rectal administration of NSAIDs should be used in high-risk patients due to its marked reduction in incidence post-ERCP pancreatitis. This will result in substantial medical and cost benefits.

Other anti-inflammatory agents: Glucocorticoids have been evaluated as a potential prophylactic agent in a few studies (intravenous and oral). Initial promising reports have been followed by five prospective controlled trials which have demonstrated its inefficacy in preventing post-ERCP pancreatitis[52-58]. Finally, a meta-analysis of six randomised controlled trials using intravenous or oral corticosteroids (total: 2448 patients) demonstrated that prophylactic corticosteroids did not reduce the incidence of post-ERCP pancreatitis[59].

Interleukin-10 is an anti-inflammatory cytokine that has been shown to limit the severity of acute pancreatitis in animal models. One initial study (144 patients, placebo-controlled) found the incidence of pancreatitis was reduced by a single IV dose given 30 min before ERCP (8% *vs* 24% in placebo)[60]. It was also effective for high-risk patients. However, two subsequent placebo-controlled trials (total 505 patients) did not demonstrate any efficacy[61,62].

Allopurinol has been studied for its inhibitory properties on oxygen-derived free radicals. Trials studying the effect of allopurinol on post-ERCP pancreatitis prevention have revealed conflicting results. Subsequent two meta-analyses of 10 RCTs (1554 patients and 1730 patients respectively) have concluded that allopurinol does not reduce post-ERCP pancreatitis and should be not recommended as a prophylactic agent[63,64].

Other agents studied (Adrenaline spray, pentoxifylline, platelet-activating factor acetylhidrolase, semapimod, aprepitant and risperidone) have either revealed discordant results or no effect on preventing post-ERCP pancreatitis[64-73].

Reduction of pancreatic secretion: Somatostatin and its synthetic analogue, octreotide are potent inhibitors of exocrine secretion of the pancreas. Various studies have been conducted using different dosing regimes (< 6 h, ⩾ 12 h or bolus). Andriulli *et al*[73] conducted a meta-analyses (16 studies) which concluded that somatostatin was ineffective in preventing post-ERCP pancreatitis. Two further controlled trials by Lee *et al*[74] and Chan *et al*[75] revealed conflicting results. Similar mixed results were found in studies using octreotide[76-78]. Therefore, somatostatin and octreotide are currently not recommended as a prophylactic agents.

Calcitonin has been studied and not been shown to have any prophylactic effect on pancreatic enzymes or complication rate[79,80].

Inhibition of protease activity: Protease inhibitors prevent activation of trypsin which is involved in the cascade of events leading to acute pancreatitis. Gabexate mesilate, nafamostat and ulinastatin have been studied in numerous studies. However, results of the trials have been conflicting. Some trials showed a benefit in reducing post-ERCP pancreatitis while others did not show any effect, especially in high-risk patients.

Seta *et al*[81] published a meta-analysis on 18 studies (4966 patients) evaluating the efficacy of protease inhibitors. This found that protease inhibitors showed a small risk reduction in ERCP-associated pancreatitis with high number needed to treat (34.5). Overall, the analysis concluded that there was no solid evidence to support the use of protease-inhibitors to prevent ERCP-associated complications.

A more recent meta-analysis by Yuhara *et al*[82] compared the effects of protease inhibitors and NSAIDs. This included 19 studies (nafamostat mesilate, $n = 4$ studies, NSAIDs, $n = 7$ studies and gabexate mesilate, $n = 6$ studies and ulinastatin, $n = 2$ studies). This found that nafamostat mesilate and NSAIDs had solid evidence for preventing post-ERCP pancreatitis (RR = 0.41 and RR = 0.58 respectively) while gabexate and ulinastatin were not associated with decreased risk of post-ERCP pancreatitis. These findings differed from the former meta-analysis by Seta *et al*[81] which did not distinguish between gabexate mesilate, ulinastatin and nafamostat mesilate.

Heparin has been studied for its anti-inflammatory properties with discordant results. A meta-analysis of four trials (1438 patients) demonstrated no benefit for prophylactic heparin in prevention of post-ERCP pancreatitis[83].

Magnesium sulphate (intravenous) is currently being studied as a calcium-antagonist and hence, a prophylactic agent against post-ERCP pancreatitis[84].

Reduction of sphincter-of-oddi pressure: Reducing sphincter of Oddi pressure would theoretically prevent development of post-ERCP pancreatitis.

Initial trials studying the effect of GTN (transdermal

or sublingual) showed promise[85,86] but three subsequent randomised trials demonstrated no significant preventive effect on post-ERCP pancreatitis[87-89].

Numerous other drugs have been studied with disappointing or conflicting results. These include nifedipine, botulinum toxin, lidocaine and phosphodiesterase inhibitor type 5[90].

Secretin causes relaxation of the Sphincter of Oddi and increases pancreatic secretion. Studies on secretin have revealed mixed results. In a German randomised trial studying the influence of secretin and gabexate-mesilate on ERCP-related complications, secretin was shown to have no effect on ERCP-induced hyperamylasemia[91]. On the other hand, Jowell *et al*[92] conducted a single-centre randomised placebo-controlled trial (869 patients) using intravenous secretin (16 μg) administered immediately before ERCP *vs* placebo. Secretin was found to decrease the incidence of pancreatitis (8.7% *vs* 15.1% in the placebo group, $P = 0.004$). Subgroup analysis revealed that secretin was highly protective against post-ERCP pancreatitis for patients undergoing biliary sphincterotomy (6/129 *vs* 32/132, $P < 0.001$).

Prevention of infection

Antibiotics: One old controlled study has evaluated the role of antibiotics on post-ERCP pancreatitis and found no effect on its incidence[93]. Another prospective randomised controlled trial involving 315 patients demonstrated that 2 g of ceftazidime administered intravenously 30 min before ERCP significantly reduced the incidence of post-ERCP pancreatitis (2.6% *vs* 9.4% in the control group, $P = 0.009$). However, this study was deemed of low-methodological quality due to the unclear allocation concealment (the control group received "no antibiotics" in place of placebo). Further studies are required before antibiotics can be recommended as a prophylactic agent against post-ERCP pancreatitis[94,95].

Anti-oxidants: Oxidant stress may be involved in the pathogenesis of post-ERCP pancreatitis. N-acetylcysteine and sodium selenite have both been studied in randomised controlled trials and was shown to not reduce the incidence of post-ERCP pancreatitis[96]. Beta-carotene was studied in a double-blind trial and did not reduce incidence of pancreatitis between the treatment and placebo group. However, there was some postulated protective effect of treatment with beta-carotene seen as there were no patients with severe pancreatitis, as compared to the placebo group (2.22%)[97].

A recent meta-analysis looked at of 11 randomised trials (3010 patients) using N-acetylcystein, selenite, beta-carotene, allopurinol and pentoxifylline. This concluded that anti-oxidant supplementation shows no beneficial effect on the incidence and severity of post-ERCP pancreatitis[98].

CONCLUSION

Selection of patients, good technique, and good aftercare remain the primary prevention of post-ERCP pancreatitis. Currently, rectal NSAIDs are the only pharmacological agents that have been shown to reduce the incidence of post-ERCP pancreatitis in especially in high-risk patients and is gaining wider acceptance. The other agents (protease inhibitors and anti-secretory agents) require larger multi-centre randomised trials that can control for multiple variables. ERCP techniques should be adapted according to the risk-profile of the patient. Guidewire technique eases primary biliary cannulation but has not been shown to reduce incidence of post-ERCP pancreatitis. Patient selection and stratifying risk in individual patients is vital in preventing post-ERCP pancreatitis. Manipulation should be minimised in high-risk cases. In addition, pancreatic stenting should be used in high-risk patients, particularly young female patients with suspected sphincter of Oddi dysfunction, difficult cannulation or history of post-ERCP pancreatitis.

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TOPIC HIGHLIGHT

WJGP 5th Anniversary Special Issues (3): Pancreatitis

Pathophysiology of autoimmune pancreatitis

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Abstract

Autoimmune pancreatitis (AIP) is a recently discovered form of pancreatitis and represents one of the diseases of the pancreas which can be cured and healed medically. International consensus diagnostic criteria have been developed, and the clinical phenotypes associated with the histopathologic patterns of lymphoplasmacytic sclerosing pancreatitis and idiopathic duct-centric pancreatitis should be referred to as type 1 and type 2 AIP, respectively. Most importantly, in type 1 AIP, the pancreatic manifestations are associated with other extrapancreatic disorders, resembling an immunoglobulin G4 (IgG4)-related disease. In addition, the pancreas of a patient with AIP is often infiltrated by various types of immune cells; the cluster of differentiation (CD) 4 or CD8 T lymphocytes and IgG4-bearing plasma cells have been found in the pancreatic parenchyma and other involved organs in AIP and factors regulating T-cell function may influence the development of AIP. From a genetic point of view, it has also been reported that *DRB1*0405* and *DQB1*0401* mutations are significantly more frequent in patients with AIP when compared to those with chronic calcifying pancreatitis, and that only *DQB1*0302* had a significant association with the relapse of AIP. Finally, it has been found that the polymorphic genes encoding cytotoxic T lymphocyte-associated antigen 4, a key negative regulator of the T-cell immune response, are associated with AIP in a Chinese population. Even if these data are not concordant, it is possible that physiological IgG4 responses are induced by prolonged antigen exposure and controlled by type 2 helper T cells. We reviewed the current concepts regarding the pathophysiology of this intriguing disease, focusing on the importance of the humoral and cellular immune responses.

Key words: Autoimmune disease; Immune system disease; Immunoglobulin G4; Meta-analysis; Pancreatitis; Pancreatic neoplasms

Core tip: Autoimmune pancreatitis (AIP) is a recently discovered form of pancreatitis and represents one of the diseases of the pancreas which can be cured and healed medically. Two types of AIP have been recognized: type 1 (usually associated with other extrapancreatic disorders) and type 2. The pancreas of a patient with AIP is often infiltrated by various types of immune cells, including cluster of differentiation 4-positive T cells and granulocytes in type 2 AIP or immunoglobulin G4 -producing plasma cells in type 1 AIP. We reviewed the current concepts regarding the pathophysiology of this intriguing disease, focusing on the importance of the humoral and the cellular immune responses.

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INTRODUCTION

Autoimmune pancreatitis is a recently discovered form

of pancreatitis and represents one of the diseases of the pancreas which can be cured and healed medically[1]. In recent years, several diagnostic criteria have been developed, such as those coming from Japan, South Korea, the United States and Italy[2]. The Japanese criteria are mainly based on radiological appearance while, in addition to imaging, the American and South Korean criteria are based on extra-pancreatic organ involvement and response to steroids, and the Italian diagnostic criteria are based on pathological findings. International consensus diagnostic criteria have subsequently been developed and, although a complete consensus has not yet been achieved, most experts agreed that the clinical phenotypes associated with the histopathologic patterns of lymphoplasmacytic sclerosing pancreatitis (LPSP) [Autoimmune pancreatitis (AIP) without granulocytic epithelial lesions (GELs)] and idiopathic duct-centric pancreatitis (IDCP) (AIP with GELs) should be referred to as type 1 and type 2 AIP, respectively[3]. The main characteristics of the two types of AIP are reported in Table 1. This will allow additional study of and the identification of specific markers of both forms of AIP; at present, the disease associated with IDCP can be definitively diagnosed only by histological examination since steroid trials cannot differentiate diseases associated with LPSP from those associated with IDCP. Type 1 AIP predominantly in Japan[4-6] whereas type 2 AIP was proposed and developed predominantly in Europe on the basis of its histopathological features[7]. Most importantly, in type 1 AIP the pancreatic manifestation is associated with other extrapancreatic disorders resembling an immunoglobulin G4 (IgG4)-related disease (IgG4-RD)[8,9]. We reviewed the current concepts regarding the pathophysiology of this intriguing disease, focusing on the importance of the humoral and the cellular immune responses.

PATHOGENESIS

Although both subtypes undergo remission when treated with corticosteroids[10,11], there is little agreement regarding their pathogenesis. The categorization of AIP as an autoimmune disorder is based on the observation that the disease is associated with the infiltration of immune cells into pancreatic tissue, and that the disease dramatically responds to steroid therapy. The pancreas of a patient with AIP is often infiltrated by various types of immune cells, including cluster of differentiation (CD) 4-positive T cells and granulocytes in type 2 AIP or IgG4-producing plasma cells and B-lymphocyte antigen CD20 in type 1 AIP[12].

SERUM IMMUNOLOGICAL FEATURES

Even if high circulating serum IgG4 levels have been proposed as a marker of AIP with good accuracy in differentiating between AIP and the overall controls, pancreatic cancer and other autoimmune diseases[13], other substances have also been reported in AIP. Hypergammaglobulinemia has been reported with a frequency ranging from 37% to 76%[1]. Levels of autoimmune antibodies, including antinuclear antibody, anticarboanhydrase 2, antismooth muscle antibody, antihuman lactoferrin and rheumatoid factor and pancreatic secretory trypsin inhibitor may all be elevated in a varying proportion of patients[1]. Higher concentrations of circulating leptin[14] as well as high levels of peptide AIP$_{1-7}$ which showed homology with an amino acid sequence of the plasminogen-binding protein of Helicobacter pylori and with ubiquitin-protein ligase E3 component n-recognin 2, an enzyme highly expressed in acinar cells of the pancreas have been also reported[15]. However, it is unclear whether the formation of these antibodies constitutes a pathogenetic event or whether they represent an associated epiphenomenon of AIP[16-18]. From a practical point of view, only IgG4 seems to be an interesting molecule for understanding the pathophysiology of type 1 AIP whereas altered cellular immune response is an interesting tool for understanding the pathophysiology of type 2 AIP.

IGG4: ITS PROPERTIES AND ROLE IN AIP

Human IgG subclasses are numbered according to their concentration in plasma; thus, IgG1 is the most abundant (greater than 50% of total IgG) while the amount of IgG4 is scarce, usually less than 5%. A polyclonal antiserum to one Ig class does not cross-react with other Ig classes, but antibodies to an Ig subclass will usually cross-react extensively with other subclasses of the same class. For the most part, IgG antibodies to bacterial polysaccharides belong to the human IgG2 subclass and a similar association was discovered between IgG antibodies to allergens and the IgG4 subclass[19,20].

It has been demonstrated that IgG4 antibodies are non-precipitating and behave like monovalent antibodies[21]. An unusual feature of IgG4 may explain its monovalency[22]; upon eelectrophoretic analysis, a substantial part of IgG4 was found to lack interchain disulphide bonds and, thus, to be a half-molecule of one heavy chain plus one light chain. This phenomenon was shown to be due to a single amino acid located at the site of the bond which differs between IgG1 and IgG4; a proline in IgG1 is replaced by a serine in IgG4. Mutating this serine into proline abolished the appearance of half-molecules on sodium dodecyl sulfate electrophoresis[23]. However, no half-molecules were found upon size exclusion chromatography, and this exchange process seems to be irrelevant *in vivo*.

There is also a peculiar characteristic of IgG4, namely its tendency to interact with other immunoglobulins. This has been studied in relation to the IgG rheumatoid factor[24]. IgG4 was found to possess an intrinsic affinity for IgG coated to a solid phase. This binding activity was not located in its variable domains, but in its constant domain. However, using labeled IgG4, it can be shown that IgG4 will also bind to coated IgG4. To further com-

Table 1 Epidemiological, laboratory, pathological and clinical characteristics of type 1 and type 2 autoimmune pancreatitis

	Type 1 AIP	Type 2 AIP
Age	Adult	Child and adult
Gender	Usually male	Equal
Serum IgG4 levels	Elevated	Normal
Histology	Lymphoplasmacytic sclerosing pancreatitis	Idiopathic duct-centric pancreatitis
IgG4 plasma cells	Well represented	Rare
Granulocytic epithelial lesions	Absent	Present
Relapse rate	High	Low
Extra-pancreatic lesions	IgG4-related disease: hypophysitis, pachymeningitis, perineural mass, chronic sclerosing dacryoadenitis, chronic sclerosing sialadenitis, lymphadenopathy, thyroiditis or hypothyroidism, pseudolymphoma, breast inflammatory pseudotumor or mastitis, pulmonary inflammatory pseudotumor, nodular pleuritis, chronic gastritis, Vater's ampulla pseudotumor, sclerosing cholangitis, lymphoplasmacytic sclerosing cholecystitis, hepatic inflammatory pseudotumor, autoimmune hepatitis, retroperitoneal fibrosis, periaortitis/periarteritis, inflammatory aneurysm, tubulointerstitial nephritis	Inflammatory bowel disease

AIP: Autoimmune pancreatitis; IgG4: Immunoglobulin G4.

plicate the situation, IgG4 with irrelevant specificity was found to bind to IgG4 antibody bound to its antigen[25]; this is a potential source of artifacts in analytical assays, not only for the measurement of bi-specific IgG4, but also for the measurement of the IgG4 antibody in general. In the case of the measurement of the bispecificity of IgG4, this "non-specific" binding was blocked by adding pooled immunoglobulins to the incubation buffer.

Total IgG4 levels are low in infancy and, thereafter, tend to increase; this presumably reflects a dependency on the maturity of accessory cells (macrophages, dendritic cells, *etc*.) which are important producers of interleukin (IL)-10. Moreover, some of the IL-10 effects are mediated via such accessory cells[26]. In fact, it has been suggested that AIP patients may be exposed to high doses of unknown disease-specific antigens, resulting in the activation of both Th1-type immune cells and regulatory T cells *via* IL-10[27].

The slow kinetics of IgG4-expressing cells is also reflected in IgG4-specific antibody levels. The IgG4/IgG1 ratio of antibodies to common foods is lower in infancy than in adolescence. This shift in the IgG4/IgG1 antibody may be related to the chronic stimulation requirement for IgG4 production, as previously discussed. This shift to IgG4 is, however, only partially due to an earlier appearance of IgG1 antibodies; it also reflects an earlier decline of IgG1 antibodies[28].

The requirements for the class switch to IgG4 are similar to those for IgE because both depend on IL-4/IL-13 induction[29-32]. Both are therefore considered to be part of the Th2 immune response. In relation to allergen-specific immunotherapy, it is sometimes suggested that a switch occurs from IgE production to IgG4 production. While a B cell can switch sequentially, such a sequential switch can transform an IgG4-producing B cell into an IgE-producing B cell, but not the other way around as a consequence of the sequence order in which the genes for the isotypes are arranged on the chromosome[30-32].

One of the effects of this common dependency on Th2 cells is that antigens which induce IgE responses are also good inducers of IgG4 responses. There are probably some regulatory differences before the class switch because the occurrence of IgG4 antibodies without IgE antibodies is not uncommon. One type of regulation is particularly important: the effects of IL-10 and related cytokines. Interleukin-10 interferes with the class switch[26] which affects both IgE and IgG4 production[33]. In addition, IL-10 is presumably needed to drive the differentiation of IgG4-switched B cells to IgG4-secreting plasma cells[34]. In addition to IL-10, IL-21 has also been found to increase IgG4 production *in vitro*[35,36]. Increased IL-21 production is characteristic of certain autoimmune diseases and is likely to contribute to autoantibody production as well as to the pathologic features of autoimmune disease[37]. In contrast, IL-21 may function as a co-adjuvant to enhance antibody responses and thereby facilitate host defense to malignances and infectious diseases[37]. Thus, the critical role of IL-21 in promoting humoral immune responses makes it an important focus of potential therapeutic interventions under conditions characterized by either the overproduction of pathogenic autoantibodies or the underproduction of protective antibodies.

The "modified Th2 response" was first used in relation to the antibody response to cat allergen[38], and it refers to subjects with IgG4 antibodies without demonstrable IgE antibodies. As the presence of IgG4 antibodies indicates a Th2 response, the absence of IgE antibodies is unexpected. However, this situation is quite common and it is seen in most beekeepers and in individuals having occupational exposure to protein antigens, such as rodent allergens in the animal house and/or exposure to mammalian serum albumin in the animal blood processing industry[39], and usually produces this phenotype[40,41]. Therefore, the modified Th2 response seems to be the typical response to an innocuous antigen[42,43]. It is intriguing that this type of response is not found in all situations where allergen exposure does not

result in IgE production. This is true not only for IgG4, but also for IgG1. The presence of high-affinity IgG antibodies (IgG1 and/or IgG4) to pollen- or mite allergens is much more common in subjects with allergen-specific IgE than in IgE-negative subjects; this difference is more marked for some allergens than for others which suggests that not all allergens are equal. However, some allergens do not induce an IgG antibody response at all whereas others induce an IgG (IgG1 and/or IgG4) response without IgE[44].

An important aspect of the IgG4 response is the slow manifestation of IgG4 antibodies. It usually takes many months of repeated antigen exposure before IgG4 responses become prominent. This is well known in the sequential analysis of sera from novice bee-keepers[45], and the analysis of sequential samples from patients who received subcutaneous allergen-specific immunotherapy shows the same pattern. It is likely that the production of sufficient IL-10 is a rate-limiting step.

CELLULAR IMMUNE ACTIVATION

CD4 or CD8 T lymphocytes and IgG4-bearing plasma cells have been found in the pancreatic parenchyma and other involved organs in AIP[46-49]. It seems that factors regulating T-cell function influence the development of AIP. The cytotoxic T-lymphocyte antigen 4 gene is an inhibitory receptor expressed on the cell surface of activated memory T cells and on $CD4^+$ $CD25^+$ regulatory T cells, and acts largely as a negative regulator of T-cell responses[50]. CTLA4 may modulate positive T-cell costimulatory signals by competing with the CD28 molecule for engagement with the B7 molecules CD80 and CD86 localized on antigen-presenting cells. In addition, and *CTLA4* + 49A/G single nucleotide polymorphisms (SNPs) have been associated with susceptibility to autoimmune diseases, such as type 1 diabetes, autoimmune thyroid disease, autoimmune hepatitis, and primary biliary cirrhosis[50]. Another form of *CTLA4*, secreted by resting T cells, can suppress T-cell activation and this soluble isoform of *CTLA4* (s*CTLA4*) is present in human serum, and it is elevated in patients with autoimmune diseases, such as autoimmune thyroid disease[51], systemic lupus erythematosus[52], and myasthenia gravis[53]. The + 6230G/A SNP in the 3' untranslated region of *CTLA4* has been also found in Graves' disease, type 1 diabetes[54] and Umemura *et al*[55] have demonstrated that AIP is closely associated with the *CTLA4* + 6230 SNP and serum sCTLA4 levels and that *CTLA4* gene plays an important role in the pathogenesis of AIP.

It has also been reported that *DRB1*0405* and *DQB1*0401* mutations are significantly more frequent in patients with AIP when compared to those with chronic calcifying pancreatitis[56], even if these initial and promising findings were not confirmed by two recent studies[57,58]. Furthermore, Park *et al*[57] found that only *DQB1*0302* had a significant association with the relapse of AIP. Finally, it has been found that the polymorphic genes (CTLA-4 49A polymorphism and -318C/+ 49A/CT60G haplotype) encoding cytotoxic T lymphocyte-associated antigen 4, a key negative regulator of the T-cell immune response, are associated with AIP in a Chinese population[59]. Even if these data are not concordant, it is possible that physiological IgG4 responses are induced by prolonged antigen exposure and controlled by type 2 helper T cells[18]. A possible explanation may come from genetically-modified animals which were produced to mimic AIP.

ANIMAL MODELS FOR STUDYING THE PATHOPHYSIOLOGY OF AIP

We believe that due the no high incidence of AIP, the animal models are important in helping the researchers to test new pathogenetic hypotheses on AIP and also new drugs able to treat this disease.

It has been demonstrated that MRL/Mp mice develop a form of autoimmune pancreatitis and that the administration of polyinosinic: polycytidylic acid may substantially shorten the time course and increase the frequency of both pancreatitis and biliary involvement[60]. The experimental model for inducing inflammatory bowel disease, *i.e.*, *IL-10*$^{-/-}$ mice, has been also used for developing type I AIP[61]. It is also possible induce AIP by immunization with lactoferrin, carbonic anhydrase or other antigens, or by alterations to the intestinal flora[62,63]. When applied, these models may answer the question regarding the first events which may lead to AIP, and to test novel therapeutic modalities, especially those regarding cellular immune activation.

CONCLUSION

Several questions remain open in the pathophysiology of AIP. The interrelationship with allergies and the multivisceral involvement in patients with AIP should be better evaluated in order to answer the question of whether IgG4 disease is initiated by allergens. In this respect, we would point out that we have recently reported an association between anisakis infection and AIP[64], and worms are well-known inducers of allergic phenomena [31]. Finally, we also need to investigate whether type 1 or type 2 AIP are different diseases or different presentation of the same illness.

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REVIEW

Intestinal barrier: A gentlemen's agreement between microbiota and immunity

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Abstract

Our body is colonized by more than a hundred trillion commensals, represented by viruses, bacteria and fungi. This complex interaction has shown that the microbiome system contributes to the host's adaptation to its environment, providing genes and functionality that give flexibility of diet and modulate the immune system in order not to reject these symbionts. In the intestine, specifically, the microbiota helps developing organ structures, participates of the metabolism of nutrients and induces immunity. Certain components of the microbiota have been shown to trigger inflammatory responses, whereas others, anti-inflammatory responses. The diversity and the composition of the microbiota, thus, play a key role in the maintenance of intestinal homeostasis and explain partially the link between intestinal microbiota changes and gut-related disorders in humans. Tight junction proteins are key molecules for determination of the paracellular permeability. In the context of intestinal inflammatory diseases, the intestinal barrier is compromised, and decreased expression and differential distribution of tight junction proteins is observed. It is still unclear what is the nature of the luminal or mucosal factors that affect the tight junction proteins function, but the modulation of the immune cells found in the intestinal lamina propria is hypothesized as having a role in this modulation. In this review, we provide an overview of the current understanding of the interaction of the gut microbiota with the immune system in the development and maintenance of the intestinal barrier.

Key words: Microbiota; Immune system; Lamina propria; Intestinal barrier

Core tip: Each of our bodies is colonized by more than a hundred trillion commensals, which include viruses, bacteria and fungi. The association between microbiota and their hosts is complex and has important repercussions for both. The diversity and the composition of the microbiota thus play a key role in the maintenance of intestinal homeostasis and the induction of immunity. These features partially explain the link between alterations in intestinal microbiota and gut-related disorders in humans. In this review, we provide an overview of the current understanding of the interaction between gut microbiota and the immune system in the development and maintenance of the intestinal barrier.

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INTRODUCTION

Each of our bodies is colonized by many commensals, such as viruses, bacteria and fungi, which are called microbiota. If we consider only the bacterial fraction,

we will be examining more than a hundred trillion cells, spread all over our skin and mucosal surfaces. This quantity makes explicit the clear mutual benefit for both the microbiota and the host[1]. Due to the complex and specific demands of symbiotic and commensal organisms to survive, it is quite difficult to culture them in the lab and, therefore, to understand their contribution to the host's biological processes. However, with current genomic sequencing techniques, a significantly greater understanding of the microbiome has been achieved.

It has become clear that adaptation of the host is influenced by the microbiome, adding new genes and functions that allow flexibility in the diet, which explains why so much effort is spent by the immune system to balance this genetic modulation. Therefore, it is reasonable to state that the increased capacity of accommodating new symbionts correlates with the increasing of the complexity of diet[2].

Although the microbiota may encompass both Eukarya and Archaea members, their relative abundance in their niche is low compared to bacteria. The highest number and most diverse microbial population is found in the colon, where there are 10^{10}-10^{12} organisms per gram of luminal content[3]. Most of the bacteria found in the colon belong to the phyla Proteobacteria, Bacteroidetes, Firmicutes, Actinobacteria, and Verrucomicrobia[4]. The relationship between microbiota and host is complex, having important repercussions for both. It is now understood that microbiota contribute to physiological processes of the host, whereas the host provides the necessary nutritional environment for its survival[1].

Interestingly, in the host's gastrointestinal tract, microbiota may have different effects. The microbiome has an important role in facilitating the development of gut-associated lymphoid tissues and participating in the metabolism of nutrients. On the other hand, under certain circumstances, the microbiota can also trigger diseases in genetically susceptible individuals[5]. Recent studies have suggested that commensal microbiota influence the host's intestinal immune response[1,6,7]. For example, certain components of the gut microbiota are capable of inducing immunoglobulin A (IgA)-mediated responses and developing Th1/Th17 effector T cells and regulatory T (Treg) cells[8-12]. Moreover, *Bacteroides fragilis* mediates the development of Foxp3$^+$ Treg cells through the activation of Toll-like receptor (TLR)2[13-15]. In the large intestine, *Clostridium* species induce Foxp3$^+$ Treg cells independently of TLRs through the induction of transforming growth factor-β (TGF-β)[16]. Thus, various types of bacteria influence intestinal T cell development.

Moreover, gut microbiota have an important role in the development of Foxp3$^+$ Treg-mediated CD4$^+$ T cell homeostasis[17] and in the acquisition of antigen repertoire of the Foxp3$^+$ Treg cells[18]. Although the mechanism is not clear, other cells from the immune system have important roles in the maintenance of the intestinal homeostasis[19]. Tr1 cells, for instance, do not express Foxp3 transcription factor and are induced by cytokines such as interleukin (IL)-10 and IL-27[20,21], which can be produced by CD103$^+$ dendritic cells (DCs) when exposed to *Bifidobacterium breve* (*B. breve*)[22]. However, the mechanism by which CD103$^+$ CX$_3$CR1$^-$ DCs sense *B. breve* is not clear because CX$_3$CR1 is required for dendrite extension[22].

In this review, we provide an overview of the current understanding of the role of the gut microbiome in the development and maintenance of the intestinal barrier.

DISTINGUISHING ENEMIES AND FRIENDS: A VISCERAL CHALLENGE

Interestingly, the intestinal immune system is able to distinguish commensals from pathogenic microorganisms. Hosts can sense commensals differently than pathogens even though they have the same immunostimulatory molecules as pathogenic bacteria and are capable of triggering inflammation if they penetrate the intestinal epithelial barrier. Many studies have shown that this sensing of commensals is important for the development and functionality of the immune system because germ-free mice have reduced cellularity and impaired functionality of the immune system in the lamina propria of the small intestine[23].

Under normal conditions, the immune system is instructed by commensal microbiota to not respond to luminal antigens. Furthermore, commensal microbiota secrete metabolites by nutrient processing, prevent infections by pathogenic microbes, provide signals to induce healthy immune development, and stimulate innate and adaptive immune responses to maintain homeostasis. However, when dysbiosis occurs, non-invasive bacteria are transported to key immune inductive sites, the mesenteric lymph nodes (MLN)[24-30]. This abnormal situation leads to aberrant immune responses against microorganisms that otherwise would not be considered a threat.

The most important difference that distinguishes pathogens from commensals is the outcome of their interaction with the host. In the intestine, an infectious process usually starts with adhesion to the brush border of intestinal cells[31,32]. After the adhesion phase, pathogenic bacteria produce virulence factors that are secreted in the external environment or injected into the cytosol of host cells. Non-invasive bacterial pathogens are able to inject virulence factors that contribute to the remodeling of the cytoskeleton of the host, leading to the formation of pedestal structures, which facilitate enhanced adhesion. Other pathogens include invasive and facultative intracellular bacteria, which secrete virulence factors that enable these pathogens to cross the epithelial barrier[33] by remodeling the actin cytoskeleton. Thus, these bacteria are able to penetrate into host cells and form a specialized niche that increases their survival[34]. Importantly, invasive pathogens need to resist innate immune defenses, survive phagocytosis and, in some cases, manipulate adaptive immunity to cross the epithelial barrier and establish infection.

Certain components of the microbiota have been shown to lead to inflammatory responses, whereas others lead to anti-inflammatory mechanisms. The diversity and the composition of the microbiota thus play key roles in the maintenance of intestinal homeostasis and partially explain the link between intestinal microbiota changes and gut-related disorders in humans[3,12,13,16,35-37].

Indeed, an association has been established between changes in the relative abundance of certain bacterial groups and the unexpected responses of the human immune system leading to diseases. The opposite situation is also observed, in which introducing a bacterial type restores homeostasis[38]. For example, *Faecalibacterium prausnitzii*, a member of the normal human microbiota, has been associated with the extension of the period of remission in patients with Crohn's disease[39].

Gram-positive bacteria have microbe-associated molecular patterns (MAMPs), such as cell wall polysaccharides, peptidoglycans, lipoprotein anchors, lipoteichoic acids (LTA) and wall bound teichoic acids (WTA), that are capable of influencing pattern recognition receptor (PRR) recognition of known MAMPs, leading, for instance, to a shield effect[40,41]. These MAMPs interact with PRRs, such as theTLRs, C-type lectin receptors (CLRs) and nucleotide oligomerization domain (NOD)-like receptors (NLRs), driving the induction of innate immune responses, with immune activation, antigen presentation, and expression of antimicrobial factors[42,43].

Commensal bacterial components are usually recognized by TLRs, which is important for protection against gut injury and associated mortality. Impairment in the interaction between commensal bacteria and TLRs have been reported to promote chronic inflammation and tissue damage, *e.g.*, inflammatory bowel disease[44]. There are two possible mechanisms by which TLR activation mediates this interaction: (1) steady-state induction of protective factors *via* constitutive detection of lumen-derived microbial products by TLR2 expressed on colonic epithelium or (2) upon epithelial damage, commensal-derived TLR ligands induce the production of protective factors. Recent studies have shown a role for CpG DNA, which is an agonist of TLR9, in mediating the beneficial effects of probiotics in the gastrointestinal tract[28].

Interestingly, a study has shown that non-pathogenic bacteria may modify immune responses by activating peroxisome proliferator-activated receptor gamma (PPARγ), a protein that promotes the export of the nuclear factor kappa B (NF-κB) subunit RelA from the nucleus to the cytosol, downregulating the transcriptional activity of NF-κB[45]. For instance, *Bacteroides thetaiotaomicron* induces PPARγ expression, leading to an anti-inflammatory profile in the intestinal compartment. This effect was not observed with a related strain, *B. vulgatus*[45]. It has also been suggested that commensal bacteria induce the expression of PPARγ through activation of the TLR4 pathway[46]. Additionally, the administration of an exogenous source of PPARγ by local gene therapy results in decreased inflammation in an experimental colitis model[47].

Another interesting mechanism by which commensal bacteria inhibit the NF-κB pathway occurs through stabilization of IκBα, a key inhibitor of the NF-κB pathway. Studies have shown that certain strains of bacteria, such as nonpathogenic *Salmonella* and *Lactobacillus casei*, inhibit IκBα degradation by the ubiquitin/proteasome system[48,49].

Although MAMPs appear to be identical between different species, there are variations in their chemical structure in regards to polymer composition, length and substitutions[4]. Some studies in several lactobacilli have targeted the D-alanylation of LTA as having a role in the immunogenicity of these MAMPs. Loss of D-alanylation of LTA in *Lactobacillus plantarum*, for instance, leads to a decrease in the capacity of the molecule to initiate TLR2-dependent proinflammatory responses[50]. In a mouse model of colitis, this mutation leads to a more protected phenotype compared with the WT[50]. In addition, other strain- or species-specific variations in the chemical modification (acetylation or pyruvylation) of the conserved peptidoglycan polymer backbones may lead to altered immunomodulatory capacities in the intestine[51].

The pilin-encoding *spaABC* operon found in probiotic *Lactobacillus rhamnosus* (LGG) leads to the production of SpaC protein, which can bind to mucus, explaining why it is more persistent in the human intestine than a closely related strain *L. rhamnosus* that lacks pili. Other protein effector molecules produced by LGG have been identified that prevent apoptosis induced by proinflammatory cytokines[52-54].

Another study demonstrated that protein glycosylation of the S-layer protein produced by *Lactobacillus acidophilus* (*L. acidophilus*) North Carolina Food Microbiology is essential for its interaction with the CLR DC-SIGN (DC-specific ICAM3-grabbing non-integrin) as it influences cytokine response in DCs and T cell priming[55].

The absence of the microbiota in germ-free mice causes developmental defects in the immune system. These mice have fewer plasma cells and intraepithelial lymphocytes, lower IgA levels, and smaller Peyer's patches and MLNs than conventional animals and exhibit increased susceptibility to pathogenic bacteria[56].

The intestinal epithelial barrier is composed of tightly attached epithelial cells, antimicrobial products, and a mucus layer. Commensal microbiota maintain the integrity of epithelial cells, stimulate them to secrete mucus and anti-microbial peptides, and thereby contribute to maintaining a basal level of steady-state host defense. Goblet cells secrete mucin-2, which forms a net-like mucus layer that physically separates most of the microbiota from the epithelium. In the colon, the lower layer is dense, relatively free of bacteria, and has concentrated levels of alpha-defensins; the upper layer contains some commensal bacteria. In the small intestine, the mucus is only one layer thick, and the epithelium is protected from microbiota by antibacterial proteins such as primarily regenerating islet-derived 3-gamma (RegⅢγ)[57].

In the innate immunity scenario, antimicrobial peptides, such as alpha-defensins, lysozyme C, phospholi-

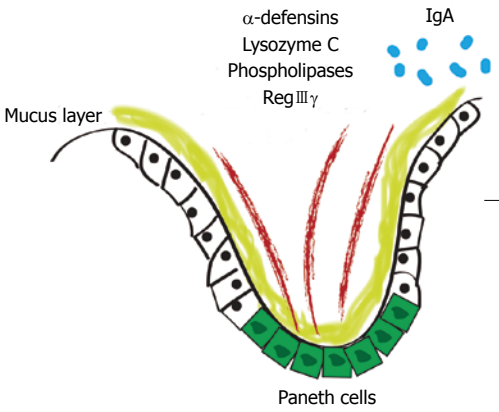

Figure 1 First line of defense of the intestinal barrier shapes the gut microbiota. Antimicrobial peptides are produced by Paneth cells, such as alpha-defensins, lysozyme C, phospholipases and C-type lectin, primarily regenerating islet-derived 3-gamma (RegIIIγ) or by enterocytes (RegIIIγ). In the adaptive immunity scenario, system effectors are secreted into the intestinal lumen, restricting bacterial penetration into the host mucus and mucosal tissue.

pases, C-type lectin, and RegIIIγ are produced by Paneth cells or by enterocytes[1,58]. In the adaptive immunity scenario, system effectors are secreted into the intestinal lumen, restricting bacterial penetration into the host's mucosal tissue. An example of this is IgA[59]. With these peptides, the host shapes the gut microbiome and controls the interaction between the host and microbiota (Figure 1).

INTESTINAL DENDRITIC CELLS AND MACROPHAGES: A COMPLEX DISTINCTION

Mononuclear phagocytes such as macrophages and DCs are the main cells involved in the maintenance of tissue integrity as well as in the initiation and control of innate and adaptive immune responses. Thus, they are crucial[60] to preserving homeostasis and preventing infections through the maintenance of tolerance to dietary antigens and control of commensal microorganisms and pathogens in the intestinal mucosa[61]. These phagocytes are distributed in lymphoid organs such as Peyer's patches and MLNs and are also very abundant in the gut lamina propria[62], but their phenotypic characterization is not completely understood.

DC populations definition was initially proposed based on the expression of the markers CX_3CR1 (fractalkine receptor) and CD103 (αE integrin)[63], but the complexity of markers has increased over time. Rivollier *et al*[64] has shown that $CD11c^+$ DCs can be divided into three populations: $CD103^+CX_3CR1^-CD11b^-$ DCs, $CD103^+CX_3CR1^-CD11b^+$ DCs, and $CD103^-CX_3CR1^{int}CD11b^+$ DCs. Particularly, $CD103^+CX_3CR1^-$ DCs. These three populations, which also express CD11c and major histocompatibility complex Ⅱ (MHC Ⅱ), have been well characterized[61,65] and have generated great interest. Currently, there appears to be a consensus that $CD11c^+CD103^+$ MHC $Ⅱ^+$ cells are the "bona fide" DCs of the lamina propria[66] because of their contribution to intestinal health, as described below.

DCs constantly survey the microenvironment and coordinate a balance of maintaining immune tolerance to harmless antigens while mounting immune responses against enteric pathogens. Depending on from which bacterial strain components were derived, DCs can be stimulated, leading to either IL-12 secretion and a Th1 response, or IL-10 secretion and a Th2 response, as will be detailed below. However, a controversy remains regarding whether the CX_3CR1^- expressing cell line is DCs or macrophages. Many groups still refer to these as DCs, whereas others categorize them as mononuclear phagocytes and others as macrophages[67]. Increasing evidence has shown that there are numerous subsets of DCs and macrophages in the lamina propria[64,67].

In addition, it has been demonstrated that $CD103^+CX_3CR1^-$ DCs develop independently of macrophage colony-stimulating factor (M-CSF) but expand in response to fms-like tyrosine kinase 3 ligand and GM-CSF[68]. These DCs appear to be the primary, if not the only, population of DCs that migrate to the MLNs through a CCR7-dependent mechanism, and they are important for the induction of oral tolerance and suppression of the development of colitis through the induction of Treg cells[62,63,68-70]. These DCs have also been described to have the ability to generate and activate $CD8^+$ T cells[71] with TGF-β production[70]. In addition, these cells produce the vitamin A metabolite retinoic acid (RA) in the gut[72]. RA production by DCs is enhanced by inflammatory stimuli and plays a role in immune homeostasis and maintenance of intestinal tolerance in the steady-state[73].

Kinnebrew *et al*[74] showed that the $CD103^+CD11b^+$ DCs from the lamina propria promote tolerance against food antigens and can rapidly produce IL-23 in response to flagellin in the lamina propria. In addition, Rivollier *et al*[64] demonstrated that in ulcerative colitis, $Ly6C^{hi}$ monocytes infiltrate into the colon and differentiate into pro-inflammatory DCs that express $CD103^-CX_3CR1^{int}CD11b^+$ and secrete high levels of IL-12, IL-23, iNOS, and tumor necrosis factor-α (TNF-α). This work showed that $Ly6C^{hi}$ monocytes have the ability to differentiate into regulatory mononuclear phagocytes or inflammatory DCs in the colon. Zigmond *et al*[75], using an acute innate model of colitis, showed that infiltrating $Ly6C^{hi}$ monocytes acquire two functionally distinct fates in the inflamed colonic lamina propria. Rather than giving rise to resident CX_3CR1^{hi} macrophages as in the healthy colon, the monocyte infiltrate initially differentiates into $CX_3CR1^{int}Ly6C^{hi}$ effector cells that sense

bacterial products *via* TLRs and NOD2. The monocyte infiltrate gives rise to a phenotypically and functionally distinct $CX_3CR1^{int}Ly6C^{lo}$ population that displays migratory DC hallmarks such as uptake and processing of orally acquired antigens and priming of naive $CD4^+$ T cells. This process occurs with C-C chemokine receptor type 7 (CCR7) expression, which enables these cells to emigrate from the colonic lamina propria towards the draining lymph nodes. Recently, Cerovic *et al*[76] demonstrated the presence of two distinct subpopulations of $CD103^-$ DCs in the intestine. Similar to what is observed in $CD103^+$ DCs, intestinal-derived $CD103^-$ DCs appear to be responsive to Flt3 and able to activate naive T lymphocytes, giving them a migratory phenotype. This presents a new mechanism for the rapid activation of T effector responses in the intestine.

In summary, $CD103^+$ DCs act as sentinels. They sense inflammatory signals, capture luminal antigens, and migrate to MLNs to interact with T cells. DCs are key players in the intestinal mucosa, promoting tolerance, and immunity. Their plasticity and motility allows them to play multiple roles as they move from the lamina propria to the epithelium and, subsequently, towards the MLNs.

In contrast, CX_3CR1^+ cells that do not express CD103 were initially described as DCs in the distal ileum. These cells were shown by several studies to play a key role in capturing and transporting intestinal antigens to MLNs[63,77,78]. Furthermore, CX_3CR1^+ cells have an ontogeny that is distinct from $CD103^+$ DCs and appear to be derived in an M-CSF-dependent manner[68]. Pro- and anti-inflammatory properties have been linked to CX_3CR1^+ cells from the lamina propria. Importantly, the CX_3CR1^+ cells from the lamina propria represent a heterogeneous group of cells, which express high and low levels of CX_3CR1[63].

CX_3CR1^{hi} cells from the lamina propria were defined as macrophages because they did not have the ability to migrate to the MLNs[61]. Therefore, CX_3CR1^+ macrophages were thereafter known as residents of the lamina propria. CX_3CR1^{hi} macrophages have been shown to contribute to intestinal homeostasis through commensal bacteria recognition and the production of anti-inflammatory cytokines[79]. The absence of CX_3CR1 led to failure to establish oral tolerance; in other words, they cannot efficiently suppress local and systemic antigen-specific immune responses upon exposure to food antigens. These cells also appear to play an important role in the induction of oral tolerance by expanding $Foxp3^+$ Treg cells[80]. Both CX_3CR1^+ macrophages and $Foxp3^+$ Treg cells are mostly abundant in the colon, whereas $Foxp3^+$ Treg cells are scarce in the duodenum. The interactions between these cells remain to be elucidated[81].

Medina-Contreras *et al*[82] demonstrated an important role for maintaining CX_3CR1^+ macrophage populations in the lamina propria preventing commensal bacteria translocation to MLNs, these cells limit Th17 responses in colitis. CX_3CR1 knockout mice (KO) had reduced frequencies of lamina propria macrophages and exhibited markedly increased translocation of commensal bacteria to MLNs. In addition, the severity of dextran sodium sulfate (DSS)-induced colitis was drastically increased in the KO compared with the control mice. These cells appear to be important for protection against intestinal inflammation and gut barrier integrity. Interestingly, Diehl *et al*[83] showed that the CX_3CR1^{hi} mononuclear phagocytes of the intestine, which had previously been shown to be non-migratory, were able to migrate into MLNs in the absence of MyD88 or under conditions of antibiotic-induced dysbiosis in a CCR7-dependent manner, carrying non-invasive bacteria captured from the intestinal lumen and inducing both T lymphocyte responses and IgA production to avoid inflammatory bowel disease. The microbiota seem to instruct the immune system to inhibit migration of bacteria to MLNs *via* CX_3CR1^{hi} cells. This mechanism leads to tolerance to commensal bacteria. Recently, using the expression of CD64, Tamoutounour *et al*[84] also managed to distinguish macrophages from DCs in the lamina propria and in the MLNs. The authors identified the gamma chain IgG receptor high affinity FcγRI (CD64) as a marker to label intestinal macrophages. The authors showed that macrophages and DCs could clearly be discriminated by CD64 expression, even when the macrophages express $CD11c^{int}$ ($CD64^+$) or when the DCs express CX_3CR1^{int} ($CD64^-$). The expression of CD64 in macrophages is induced by interferon (IFN)-γ and suppressed by IL-4. However, on the other hand, IL-10 also upregulates CD64 and might sustain CD64 expression on macrophages. In the last stage of development in the lamina propria, macrophages express $CD64^+CD11b^+CX_3CR1^{hi}$. More importantly, it has been demonstrated that CD64 can be used as a reliable marker of macrophages in both the small and large intestine under steady-state conditions and inflammatory responses[85].

CX_3CR1^+ macrophages and $CD103^+$ DCs in the intestinal lamina propria have developed mechanisms to prevent exacerbated responses to commensal bacteria, but they can also respond to infection by pathogens. The effects of gut microbiota in the cells of the lamina propria, which are crucial in recognizing bacterial tolerance induction and orientation of T cell responses, appear to be essential for the maintenance of intestinal immune homeostasis. The plasticity of dendritic cells, for example, is extremely important for their ability to respond to microbial stimuli and the ability to capture luminal bacteria and migrate to MLN. In the lamina propria, macrophages are educated to acquire non-inflammatory characteristics. Interestingly, however, the expression of CX_3CR1^+ in macrophages that were isolated from colon differs considerably from those isolated from the duodenum, jejunum and ileum, suggesting that the instructions that macrophages receive from these regions are variable. This makes it clear that distinct commensal populations in different regions of the intestine give signals to these cells, influencing their profiles[86].

The role of gut microbiota in macrophage and DC development is not clear. It is known that these cells participate in the regulation of intestinal immune responses

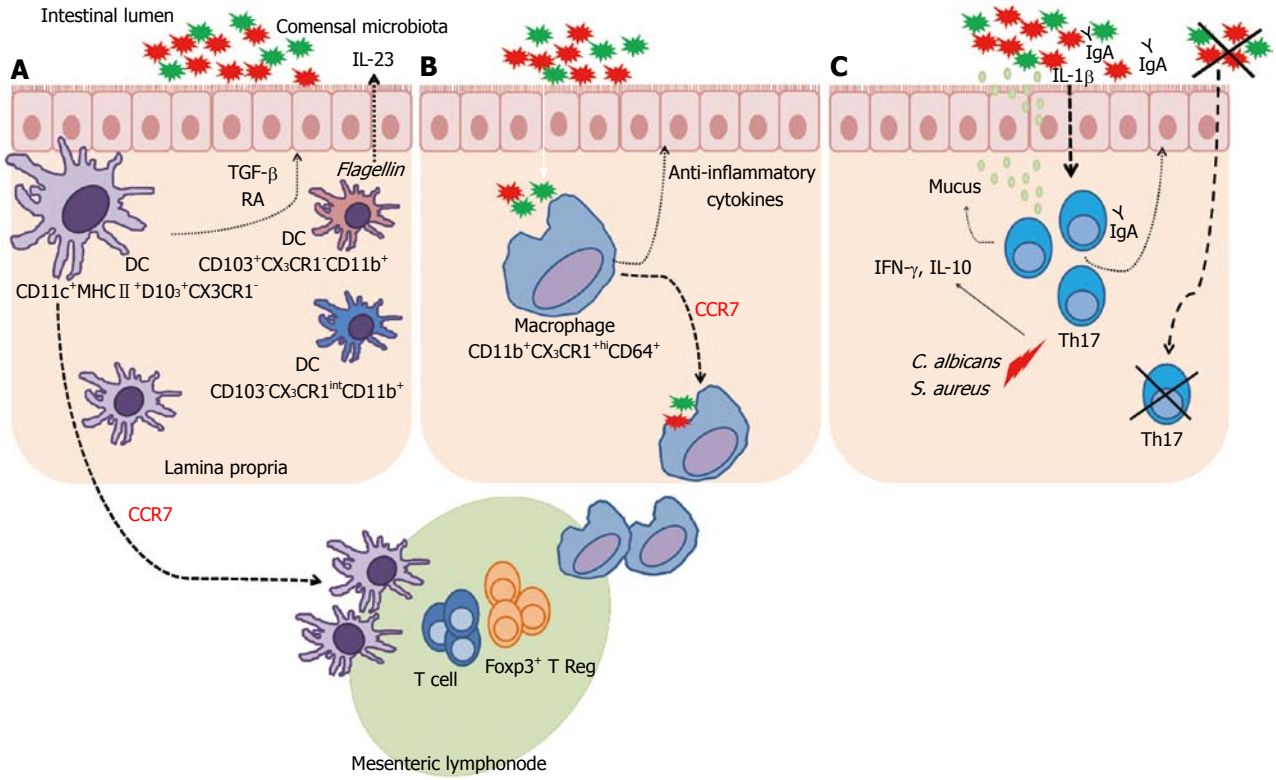

Figure 2 Complex interaction between microbiota, dendritic cells and macrophages. A: $CD11c^{+}MHC\,II^{+}D103^{+}CX_3CR1$ cells migrate to the mesenteric lymph nodes (MLN) through a C-C chemokine receptor type 7 (CCR7) dependent mechanism. These dendritic cells (DCs) can generate and activate $CD8^{+}$ T cells and Treg $Foxp3^{+}$ cells. These DCs also have the ability to produce transforming growth factor-β (TGF-β) and retinoic acid (RA). $CD103^{+}CX_3CR1^{-}CD11b^{+}$ DCs can produce interleukin (IL)-23 in response to flagellin. Other DCs in lamina propria are $CD103^{-}CX_3CR1^{int}CD11b^{+}$; B: $CD11b^{+}CX_3CR1^{hi}CD64^{+}$ macrophages in the lamina propria contribute to the intestinal homeostasis through the production of anti-inflammatory cytokines and are able to recognize commensal microbiota beyond the epithelial barrier. These macrophages had previously been described as non-migratory were able to migrate into the MLNs, in a CCR7-dependent manner, carrying non-invasive bacteria captured in the intestinal lumen induces T lymphocyte responses; C: Under steady state, Th17 cells are usually found in the lamina propria of the small intestine, where its development depends on the presence of dietary antigens and commensal microbiota. These cells have important effects on the intestinal epithelium, improving the barrier function by stimulating mucin production, function of tight junction proteins, and increasing the transport of IgA to lumen. Candida albicans and Staphylococcus aureus induce Th17 cells, which produce interferon (IFN)-γ and IL-10. In the absence of microbiota, Th17 cells are not found. IL-1β, induced by commensal bacteria, is critical for differentiation of Th17 cells in the intestine.

against various microorganisms and diseases by producing several pro- and anti-inflammatory cytokines in an attempt to maintain intestinal homeostasis. This is an important topic for further investigation. A summary of these findings is illustrated in Figure 2A and B.

MICROBIOTA AND ITS ROLE ON TH17 ACTIVATION

Th17 cells are a prominent population among the T cells present in the intestinal lamina propria that cooperate in maintaining intestinal homeostasis[87]. These Th17 cells play a key role in mucosal host defenses as well as in the development of autoimmune diseases[88]. Under steady-state conditions, Th17 cells are usually found in the lamina propria of the small intestine, where Th17 cells development depends on the presence of dietary antigens and commensal flora[12]. These cells are a subset of $CD4^{+}$ T cells and primarily secrete IL-17, which has important effects on the intestinal epithelium, through improving the barrier function and stimulating mucin production, as well as on the function of tight junctions and transport of IgA to the lumen[89,90].

While accumulating evidence shows that Th17 cells play a role in the pathogenesis of a variety of inflammatory conditions, there is considerable controversy concerning whether they also contribute to the maintenance of intestinal immune homeostasis. Both protective and pathogenic roles of IL-17 have been reported in patients with inflammatory bowel disease (IBD) and in experimental colitis in mice[91,92]. Patients with IBD often have increased levels of IL-17, and IL-17 specific inhibition protected them from this disease[93].

It is important to note that during inflammatory conditions, such as experimental autoimmune encephalomyelitis (EAE), the induction of Th17 cells requires the following cytokines: IL-1β, IL-6, IL-23 and TGF-β1[88]. In addition to being present during the inflammatory response, a population of T cells that expresses retinoic acid receptor, RORγT (which is a specific transcription factor of Th17 cells), was also found under steady-state conditions (sTh17) in the lamina propria of the small intestine[94], where they accumulate in the presence of luminal commensal microbiota.

An important role for these cells in the digestive

tract has been shown in RORγT KO mice, which lack both innate and Th17 cells. These mice displayed a large expansion of lymphoid follicles in the intestine, had an increased number of Th1 and IgG^+ B cells and were extremely susceptible to DSS-induced colitis[95]. Moreover, Th17 cells are not found in the gastrointestinal tract of germ-free mice, suggesting that this cell population is generated in response to the gut microbiota[96]. Segmented filamentous bacteria (SFB) are potent inducers of Th17 in the intestine, despite being found in low frequency in the intestine[12]. Other components of the microbiota can also stimulate Th17 cells in the intestine, including the "Altered Schaedler Flora" (ASF), which comprises *L. acidophilus* (strain ASF 360), *Lactobacillus salivarius* (strain ASF 361), and *Bacteroides distasonis* (strain ASF 519) and several other species[14]. This stimulation depends on the host immune response and the exposure time. The induction of Th17 cells in the intestinal lamina propria by SFB protects against *Citrobacter* infection by stimulating the production of Reg Ⅲγ defensins[12]. Nevertheless, SFB also increases the susceptibility to EAE, arthritis[97], colitis[98] and diabetes[99]. The exact mechanism by which SFB are able to induce Th17 differentiation in the intestine is not understood. Flagellins are potentially involved[100]. Colonization with SFB leads to increased IgA production and secretion; moreover, the colonization of germ-free mice with SFB also increases the expression of Th17 cells in the intestine[12,101].

A recent study has shown that *Candida albicans* and *Staphylococcus aureus* induce the expression of Th17 cells and that these cells are able to produce IFN-γ and IL-10[102]. Furthermore, Shaw *et al*[103] showed that IL-1β induced by commensal bacteria is critical for the differentiation of Th17 cells in the intestine under steady-state conditions. It is clear that the differentiation of Th17 cells is extremely complex and triggered by various ligands, such as microbial cells and innate cytokines. Th17 cells are double-edged swords: they can act as both protectors and aggressors, depending on the context. They are generated in response to microbiota, and they are able to induce the secretion of pro- and anti-inflammatory cytokines with important effects on the intestine epithelium. Th17 cells are also important for maintaining homeostasis between the host and microbiota. A summary of these findings is illustrated in Figure 2C.

GUT PERMEABILITY: AN UNCLEAR CONNECTION BETWEEN ALTERED GUT MICROBIOTA AND THE IMMUNE SYSTEM

The gastrointestinal tract is considered the largest surface of the human body that is in contact with the environment. The mucosal barrier plays an important role in the selection of luminal factors that are allowed to enter the body and those that are forbidden to enter because of the danger they may pose.

The mucosal barrier is composed of a mucus layer, epithelial cells, and intercellular tight-junction proteins between these cells[104]. Tight junction proteins are key molecules for determining paracellular permeability; they form complex protein systems, which are organized by the transmembrane proteins occludin and claudins interacting with zonula occludens proteins that bind to the actin cytoskeleton. When actin contracts, it leads to increased permeability to electrolytes and small molecules[105].

In the context of inflammatory bowel syndrome (IBS), some studies have shown that the intestinal barrier is compromised, and decreased expression and differential distribution of tight-junction proteins are observed[106-110]. The nature of the luminal or mucosal factors that affect the function of tight junction proteins is still unclear.

There is some evidence suggesting a role for the mast cell enzyme tryptase in the degradation of the tight-junction proteins and increased permeability because the infiltration and activation of these cells are increased in IBS patients in association with higher output of tryptase from their mucosal biopsies[111]. Therefore, it is possible that these proteins are both expressed less because of transcriptional/translational regulation and destroyed because of increased tryptase output. Understanding the predominant mechanism involved may present a possibility for interference as a potential therapy by improving the intestinal barrier in IBS. However, it is still unclear whether the altered gut microbiota found in IBS or the modulation of intestinal immune cells may trigger detrimental effects on the gut barrier. Recent findings suggest that there is a complex interaction between alterations in microbiota and immune cell recruitment, which lead to physiological responses such as an altered gut barrier.

Some probiotic molecules appear to modulate changes in host cell signaling. This scenario can be illustrated by the p40 and p75 proteins produced by LGG: they co-modulate phosphoinositide 3-kinase (PI3K)/Akt signaling[112]. When TNF-α, IL-1β and IFN-γ are secreted, p40 protein and unidentified epidermal growth factor receptor ligands stimulate the production of Bcl2, stabilizing tight-junction proteins and promoting epithelial barrier function and cell survival[113].

TLR and NLR signaling triggered by MAMPs are likely to have roles in the production of physical and chemical defenses in the small intestine, limiting the numbers of mucosa-associated bacteria and preventing bacterial penetration of host tissues. Some bacterial strains can also stimulate regulatory immune mechanisms through the activation of DCs and $CD4^+Foxp3^+$ T cells[114]. This phenomenon has been shown by a study in which *Bifidobacterium breve* led to the induction of IL-10-producing regulatory Tr1 cells in the colon *via* TLR2/MYD88-dependent production of IL-10 and IL-27 in $CD103^+$ DCs[22]. WTA and LTA have also been shown to shift IL-10/IL-12 ratios in macrophages towards IL-10 *via* the TLR2/Extracellular signal-regulated kinase (ERK) signaling pathway[115].

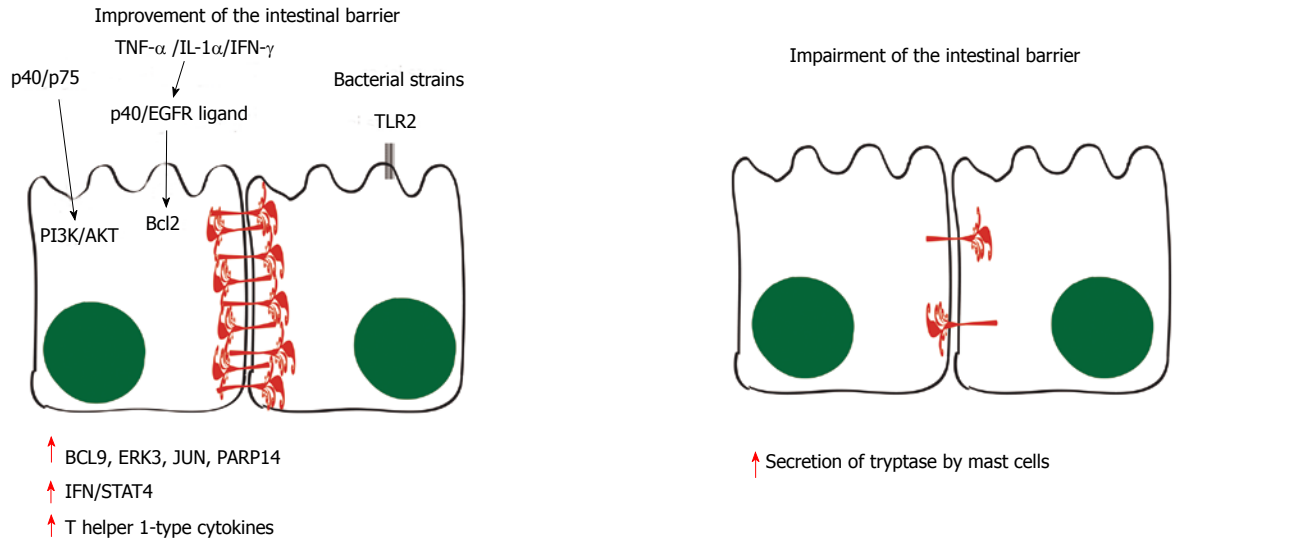

Figure 3 Pathways involved in the improvement and in the impairment of the intestinal barrier. Some probiotic molecules seem to modulate changes in host cell signaling, such as the p40 and p75 proteins, which comodulate phosphoinositide 3-kinase (PI3K)/Akt signaling. When tumor necrosis factor-α (TNF-α), interleukin (IL)-1α and interferon (IFN)-γ are secreted, the p40 protein and unidentified epidermal growth factor receptor (EGFR) ligands stimulate the production of Bcl2, stabilizing tight junctions and promoting epithelial barrier function and cell survival. Toll-like receptor (TLR) and NOD-like receptor (NLR) signaling triggered by microbe-associated molecular patterns (MAMPs) are likely to have roles in the production of physical and chemical defenses in the small intestine, limiting numbers of mucosa-associated bacteria and preventing bacterial penetration of host tissues. Moreover, BCL-9, ERK3, JUN and poly(ADP-ribose) polymerase (PARP)14 have also been implicated in the signaling events induced by probiotics, leading to induction of IFN/STAT4 pathway activation and to the production of T helper 1-type cytokines. On the other hand, evidences suggest that the mast cell tryptase is involved in the degradation of the tight-junction proteins and increased permeability, since the infiltration and activation of these cells are increased in inflammatory bowel syndrom patients in association with higher output of tryptase from their mucosal biopsies. BCL9: B-cell lymphoma-9; JNK: c-Jun N-Terminal Protein Kinase; ERK: Extracellular signal-regulated kinase.

Functional changes of epithelial cells can also be triggered by bacterial components. LGG proteins p40 and p75 increase the resilience of intestinal epithelial cells to cytokine-induced proapoptotic signals and induce a strengthening of the epithelial barrier function involving the EGFR/PI3K/Akt/PKC pathway[113]. Another study has shown that the expression of tight-junction in humans is modulated through TLR2 signaling[116]. Moreover, B-cell lymphoma-9, ERK3, c-Jun N-Terminal Protein Kinase and poly(Adenosine diphosphate-ribose) polymerase (PARP)14 have also been implicated in the signaling events induced by LGG consumption, leading to the induction of IFN/STAT4 pathway activation and production of T helper 1-type cytokines[117].

In the context of obesity and metabolic syndrome, it is unclear how immune modulation occurs in the intestine, despite numerous lines of evidence showing that intestinal barrier disruption is associated with alterations in the gut microbiota[118-121]. Conversely, other models, such as colitis and inflammatory bowel disease, have shed light on mechanisms that potentially orchestrate the modulation of the immune system by the microbiota, which may be very useful for understanding gut barrier alterations in models of obesity.

Other studies have shown that the endocannabinoid system is also involved in the regulation of the gut barrier and inflammation. Metabolic endotoxemia and systemic inflammation are suppressed by 2-arachidonoylglycerol; these phenomena are potentiated by 2-palmitoylglycerol. In addition, 2-oleoylglycerol leads to the release of gut peptides from intestinal L-cells, such as the glucagon-like peptide 2, which is associated with the regulation of gut barrier function[120].

Although some investigations have led to the hypothesis that Gram-negative bacteria may be involved in triggering metabolic endotoxemia and, therefore, in worsening the condition of the intestinal barrier[119-123], it is plausible that mechanisms other than lipopolysaccharide (LPS) are responsible for this. This is illustrated by the study that showed that *Akkermansia muciniphila*, a Gram-negative bacterium, decreased metabolic endotoxemia, which was induced by a high-fat diet, through increasing levels of endocannabinoids that control inflammation, the gut barrier and the gut peptide secretion[121]. A summary of these findings is illustrated in Figure 3.

PROBIOTICS: EPITHELIUM, IMMUNE RESPONSES AND THERAPEUTICS

Probiotics have been described as a "beneficial live microbial supplement which improves the intestinal microbial balance"[124]. The mechanisms of action of probiotics have been thoroughly discussed. It has been demonstrated that they are capable of modulating the permeability of epithelial barriers, changing the inflammatory potential of epithelial cells, or directly modulating the activity of immune cells[125-127].

The immune system of the intestinal mucosa plays a key role in defending against pathogens. The potential role of probiotics in the function of immune cells, such as DCs, suggests that certain species of probiotics could be used to modify T lymphocyte responses[128]. Certain

probiotics that have the property of inhibiting IL-12 secretion can be extremely important in Th1-mediated diseases due to their ability to restore the homeostasis of the intestinal immune system[129,130]. Probiotics have also been described as being capable of inducing Foxp3$^+$ Treg cells or developing TGF-β-bearing Treg cells[131,132]. Furthermore, stimulation of the immune system with probiotics can contribute to the production of IL-10, an essential cytokine for intestinal homeostasis maintenance[22,115,132]. Moreover, probiotics have been described as antagonists of pathogenic bacteria because they trigger effects such as reduction of luminal pH, inhibition of bacterial adherence, and production of antimicrobial molecules[4].

The use of probiotics can promote improvement in several diseases; for example, they cause diminished symptoms of IBD[124]. Most of the currently used probiotics belong to the genera *Lactobacillus* and *Bifidobacterium*. In EAE mouse models, *L. paracasei* and *L. plantarum* induced CD4$^+$ CD25$^+$ Foxp3$^+$ T cells in the mesenteric lymph nodes, leading to increased TGF-β expression and reduced inflammation in the central nervous system[132]. Other studies have confirmed this immunomodulatory effect of *Lactobacillus,* showing that it leads to augmentation of IL-10 levels[129,132,133] and to a reduction of pro-inflammatory cytokines such as IL-6 and TNF-α[134]. Other probiotics are able to inhibit NF-κB, such as *L. plantarum,* suggesting that it induces tolerance to food antigens[135].

Some studies also highlight *Lactobacillus* as an activator of conventional DCs and *Bifidobacterium* as an activator of CD103$^+$ DCs[70]. Bermudez-Brito *et al*[136] showed that *Lactobacillus paracasei* Collection Nationale de cultures de microorganismes I-4034 treatment led to a suppressed pro-inflammatory cytokine and chemokine profile in human intestinal DCs challenged with *Salmonella* in a TLR2-dependent manner. In addition, probiotics may induce functional changes in epithelial cells. It is not clear which soluble factors are released in the conditioned medium by LGG, but they are suggested as regulators of the expression of heat shock proteins 25 and 72 in intestinal epithelial cells *in vitro*[137], conferring protection against oxidative stress-mediated apoptosis. Another probiotic, *L. plantarum* WCFS1, modulates the expression of tight junction proteins in humans *via* TLR2 signaling pathways[116]. Probiotics may also lead to increased production and secretion of IgA through modulating cytokine expression in the intestine[138].

INTERACTIONS BETWEEN MICROBIOTA, THE IMMUNE SYSTEM AND ORGANS

Despite our growing understanding about the consequences of the host-microbiota interaction for the immune function in the intestine, the extent to which the intestinal flora contribute to immunity at distal sites remains an enigma.

The skin provides the first line of defense by the host immune system against invading pathogens. There are several commensal communities residing on the skin[139]; inflammatory skin diseases, such as psoriasis and dermatitis, have been associated with imbalanced skin microbiota[140,141].

Naik *et al*[142] showed that the commensal microbiota of the skin is necessary for an appropriate immune response. Protective immunity to a pathogen on the skin was considered critically dependent on the microbiota of the skin, and not of the intestine. These cutaneous commensal microorganisms exert their effects by increasing IL-1 signaling and amplifying responses according to the site of inflammation. Therefore, through their ability to promote IL-1 signaling and, thus, the function of effector T cells, commensals of the skin are likely drivers and amplifiers of pathologies of the skin[142].

Commensal bacteria, such as *Streptococcus epidermidis,* produce ligands that are capable of activating the TLR pathway. To investigate whether commensal bacteria influence the skin inflammatory response, Lai *et al*[143] treated primary human keratinocytes with a TLR ligand, poly(I:C), which was able to activate TLR3 signaling, causing an increase in the expression of TNF-α and IL-6. The authors also observed that staphylococcal lymphotoxin is a selective suppressor of TLR3-mediated inflammation in the skin.

The investigation of lung microbiota is relatively new and may lead to new discoveries about respiratory diseases. The lungs of healthy humans were previously believed to be sterile. However, studies have shown that the lungs of healthy patients are colonized by some communities of bacteria[144,145]. The results of published studies differ, but Proteobacteria, Firmicutes and Bacteroidetes are commonly identified at the phylum level. At the genus level, *Pseudomonas, Streptococcus, Prevotella, Veillonella* and *Fusobacteria* predominate with minor contributions from potential pathogens, including *Haemophilus* and *Neisseria*[146].

Low levels of bacterial products can be detected in systemically infected patients and, to a lesser degree, in healthy people, suggesting that the products of intestinal microbiota can activate TLR and NLR in the liver. Numerous studies indicate that macrophages are also sensitive to physiologically relevant levels of microbial products reaching the liver as these cells respond to low concentrations of LPS through the activation of NF-κB and production of pro-inflammatory cytokines[147].

Alteration of the permeability of the intestine is the primary means by which intestinal microbiota alterations activate innate immunity in the liver. Therefore, liver injury mediated by endotoxin can be reversed by removing Kupffer cells or by neutralizing TNF-α with anti-TNF-α antibody[148]. Recent evidence also demonstrated the involvement of microorganisms in less severe forms of liver disease. More specifically, intestinal microbiota can have a central role in liver fibrosis as evidenced by results with mice showing that chemically induced fibrosis is associated with increased bacterial translocation[149].

CONCLUSION

Understanding the interaction between commensal mi-

croorganisms and the host contributes to comprehending the functionality of a new organ, the microbiota, which is responsible for the maturation and modulation of many systems, such as the immune and metabolic systems. Although many of these microorganisms perform functions that are essential for maintaining the homeostasis of the immune system, they pose a threat if the intestinal barrier is impaired and may lead to numerous pathologies, such as inflammatory bowel disease and metabolic syndrome. Further investigations are necessary to increase the understanding of how the microbiota influence the development of the immune system and cell differentiation as well as how these changes are able to stimulate responses in distant organs.

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MINIREVIEWS

Electrophysiology as a tool to unravel the origin of pancreatic pain

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Abstract

Intense abdominal pain is the most common symptom in chronic pancreatitis, but the underlying mechanisms are not completely understood and pain management remains a significant clinical challenge. The focus of pain origin in chronic pancreatitis traditionally has been on the pancreatic gland, assuming pain to originate in the pancreas or its surrounding organs. However, research in the last decade points to abnormal central nervous system pain processing. For this reason, electroencephalography has been receiving increasing attention. In contrast to imaging methods such as functional magnetic resonance imaging and positron emission tomography, electroencephalogram has excellent temporal resolution making it possible to investigate central processing of pain on a millisecond time scale. Moreover, continuously advancing methodology made it possible to explore brain sources responsible for generation of evoked potentials and hence to study brain reorganization due to pain in chronic pancreatitis. The aim of this review is to give an overview of the current methods and findings in electroencephalography as a tool to unravel the origin of pancreatic pain.

Key words: Chronic pancreatitis; Electrophysiology; Evoked potentials; Brain source localization; Electroencephalography frequency analysis; Visceral pain; Chronic pain; Pancreatic pain

Core tip: Chronic pancreatitis (CP) is a disease with progressive destruction of the pancreatic gland and intense abdominal pain is one of its main characteristics. The understanding of pain in CP has conventionally focused on the diseased pancreas itself, assuming pain to be due to increased parenchymal or ductal pressure. However, recent research points to possible involvement of abnormal central nervous system pain processing. This review gives an insight into electrophysiology as a tool to unravel brain abnormalities underlying pancreatic pain and provides up to date electrophysiological results in this patient group.

Lelic D, Olesen SS, Graversen C, Brock C, Valeriani M, Drewes AM. Electrophysiology as a tool to unravel the origin of pancreatic pain. *World J Gastrointest Pathophysiol* 2014; 5(1): 33-39 Available from: URL: http://www.wjgnet.com/2150-5330/full/v5/i1/33.htm DOI: http://dx.doi.org/10.4291/wjgp.v5.i1.33

INTRODUCTION

Pain is a prominent symptom in chronic pancreatitis (CP), but its underlying mechanisms are incompletely under-

stood and probably multifactorial[1]. Thus, no single remedy for pain relief exists and an optimal pain treatment can only be achieved on the basis of a better understanding of the pain mechanisms underlying pain in the individual patient[2]. While, the focus of pain origin in CP historically has been on the pancreatic gland, assuming pain to originate in the pancreas or its surrounding organs, recent findings indicate that both peripheral and central pain processing are abnormal in CP patients[1,3]. Various mechanisms responsible for the altered pain processing have been proposed, including pancreatic neuropathy and neural remodeling[4,5], sensitization of neurons in the spinal cord and the brain[6,7], reorganization of the brain areas involved in visceral pain processing[8] and alterations in descending pain control from the brainstem and other supraspinal structures[9]. The diagnostic work up of patients with painful CP should therefore not only focus on pancreatic and extra pancreatic causes of pain (*e.g.*, pseudocysts, duct dilation and strictures, pancreatic head mass, *etc.*), but also include an assessment of central pain processing.

Central pain processing can be studied by various methods, but none of the current available techniques have gained wide clinical use. Neuroimaging methods based on indirect measures of neuronal activity, such as functional magnetic resonance imaging (f-MRI) (changes in haemodynamic responses) or positron emission tomography (changes in metabolic responses), have been used extensively to study pain processing[10,11]. Although these methods possess excellent spatial resolution and have greatly contributed to our knowledge of the structural basis of the pain system, their temporal resolution is relatively poor (order of several seconds). Consequently, in order to address the dynamics of pain processing (*i.e.*, the pain-specific sequential brain activation underlying pain perception), a method with high temporal resolution is needed, such as electroencephalography (EEG) which measures neuronal activity directly[12]. An additional rationale for using electrophysiological methods for objective characterization of pain processing is the relatively low cost and ease of use compared to other neuroimaging methods. This allows evaluating pain processing in the clinical setting and may in the future guide clinicians in tailoring individualized therapies.

The aim of this review is to give an overview of electrophysiology as a tool to unravel the origin of pain in patients with CP. This will be done by giving a summary of up-to-date methods and findings of research done in CP patients by means of: (1) spontaneous EEG and (2) EEG evoked potentials (EPs). The review will be concluded by giving some future perspectives for EEG research in CP patients.

SPONTANEOUS EEG IN CP

To explore the specific brain circuitry involved in chronic pain conditions such as neuropathic pain and central sensitization[13], spontaneous EEG recorded while the patient is at rest may provide a clinical useful tool to identify pain mechanisms in individual patients.

Spontaneous EEG captures the excitatory and inhibitory postsynaptic potentials, which are regulated by various homeostatic processes[14,15]. The potentials oscillate at various frequencies, which have traditionally been split into standard frequency bands such as delta (0.5-4 Hz), theta (4-8 Hz), alpha (8-12 Hz), beta (12-32 Hz), and gamma (32-80 Hz). As the oscillations are regulated by homeostatic processes, quantification of the EEG reveals important information regarding the neurotransmitters in the brain in each patient. As some of these neurotransmitters are involved in pain processing, EEG analysis can be used to study the central nervous system (CNS) state in the patient[16]. Furthermore, spontaneous EEG can be used to identify the pain mechanisms and the neuroplasticity in the CNS caused by many years of pain[17]. For an example of spontaneous EEG analysis, please see Figure 1.

Although spontaneous EEG has been applied to many patient groups[18-20], there seems to be a lack of studies in CP patients. We have compared the resting state EEG of thirty-one patients diagnosed according to the Mayo Clinic diagnostic criteria to that of fifteen healthy volunteers[21]. Delta, theta and alpha activities were increased in the patients as compared to controls. The increase in theta band activity could indicate disturbed thalamocortical interplay[22], while the increased alpha activity may reflect inhibition of sensory stimuli[23]. Delta band activity increase was lost in a sub-analysis adjusting for opioid treatment, diabetes mellitus and alcohol aetiology, indicating a less pain specific role of the slower rhythms in pain processing. A significant interaction between electrode and participant group were evident for all frequency bands and it was plausible that differences in amplitude strengths were confined to specific cortical areas. Differences were seen for most central electrodes in the theta band and this supported the hypothesis that thalamocortical dysrhythmia may play a key role in the altered pain processing in the chronic pancreatitis patients.

EPs

To explore how the pain relevant brain networks are modified due to CP, EEG pain EPs can be utilized. EEG EPs are electrical potentials recorded from the nervous system following presentation of a stimulus, such as pain on the skin or viscera. Since EP amplitudes tend to be much lower than the spontaneous EEG amplitudes, multiple stimulations are required and the corresponding EPs are averaged. During this process, amplitudes of EPs are increased, while the random background activity (spontaneous EEG) cancels out. Then, the amplitudes and latencies of EP peaks can be analysed and compared between healthy controls and patients in order to observe whether there are any alterations in the patient group. Time-frequency analysis of EPs can also

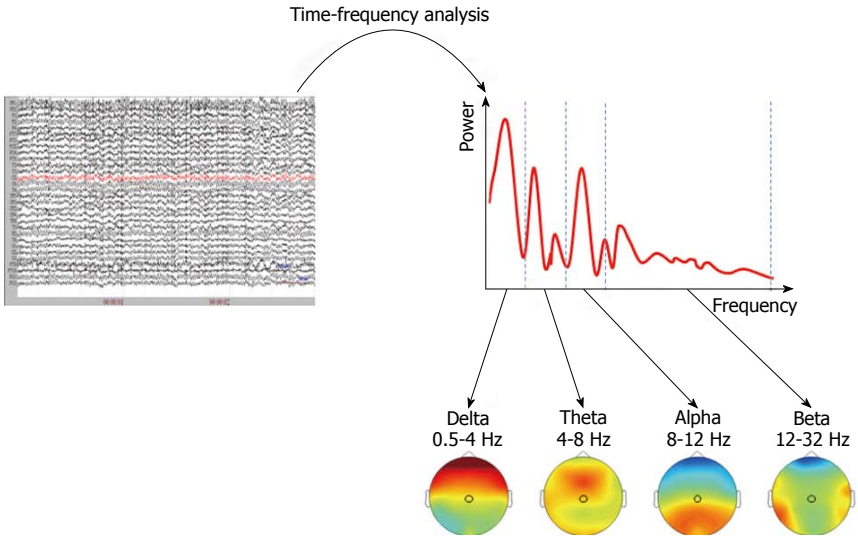

Figure 1 Spectral analysis of resting electroencephalography. The multi-channel resting electroencephalography is recorded, and pre-processed including filtering, interpolation of channels with abnormal signal shape and de-artifacting. For each channel (in this example the Cz electrode - highlighted in red), time-frequency analysis is applied to extract the spectral distribution of the signal. The distribution is summed in standard frequency bands: delta, theta, alpha and beta, and results for each band presented on topographical maps (the Cz results are indicated by a black circle). Each channel shows its own spectral distribution, with the typical pattern: delta (frontal), theta (centro-frontal), alpha (occipital), and beta (temporal).

be done as discussed in the spontaneous EEG section. Moreover, when enough of recording scalp electrodes are used to provide the full scalp coverage (typically > 30 electrodes), topographical distributions can be analysed in order to investigate at which topographical location the activity is maximal for each peak. Please see Figure 2. Recent advancements in EP analysis methods allow for looking at brain source generators of these topographies and hence it is possible to investigate where in the brain the changes due to CP are occurring on a millisecond time scale[24].

Research with EPs in CP to date is limited. Previous research mainly made use of visceral EPs, but recently somatic contact heat evoked potentials (CHEPs) have also been used. The findings of these studies are summarized in Table 1 and presented below in their respective subsections.

Visceral EPs

In a study by Dimcevski *et al*[8], where painful EPs at three stimulation sites (oesophagus, gut and duodenum) were utilised, a decrease in latencies of the EPs was seen. The authors argued that the decrease in latencies most likely reflects central nervous system changes such as hyperexcitability and reorganization. The central reorganization was confirmed by source analysis where the bilateral insular sources were localized more medial in all three stimulation sites and the cingulate source was localized more posterior in patients for the oesophageal stimulation. Since insula is suggested to play an important role in integrating visceral sensory and motor activity together with the limbic integration[25], the authors concluded that the reorganization within the insular cortex in CP patients likely mirrors disruption in the coordination and processing of visceral pain. The change within cingulate cortex, although more discrete than the change within insula, was interpreted as reflecting changes in the cognitive and effective pain components as a result of the long-lasting frequent pain attacks in CP. Another study using the same dataset for oesophageal EPs as Drewes *et al*[26] but looking at data in frequency domain was done. The authors found that patients showed higher activity in theta frequency band and theta activity was centered around 4.4 Hz in patients and 5.5 Hz in healthy controls. The authors interpreted the increased theta activity in patients as possibly reflecting a thalamocortical dysrhythmia like discussed above for the resting EEG. Additionally, the power in delta band was higher in healthy controls than in CP patients. Due to a major spread between subjects regarding delta activity, it could not be clearly concluded whether the changes in delta band were due to expected delta increase due to pain in healthy volunteers or whether the decrease in delta activity in patients is a consequence of chronic pain. Hence, further studies addressing this should be conducted. Recently, we have done several CP studies where rectal EPs were applied[9,27,28]. We observed that the rectal evoked potential latencies were prolonged and this was confined to the frontal scalp electrodes[27]. This is in contrast to the previous oesophageal CP study and this could be due to different stimulation sites. Since the prolongation of latency was only seen at the frontal electrodes, this likely reflects an alteration in cerebral pain processing. This was confirmed by analysis of underlying sources showing that insular dipoles were localised more posterior in the patients than in healthy subjects (please see Figure 3) and this shift was negatively correlated to the patient symptom scores. These findings suggest a pain-associated adaptive cortical reorganization in CP patients. In another rectal EP study, it was also seen that the first positive component (P1) was prolonged in

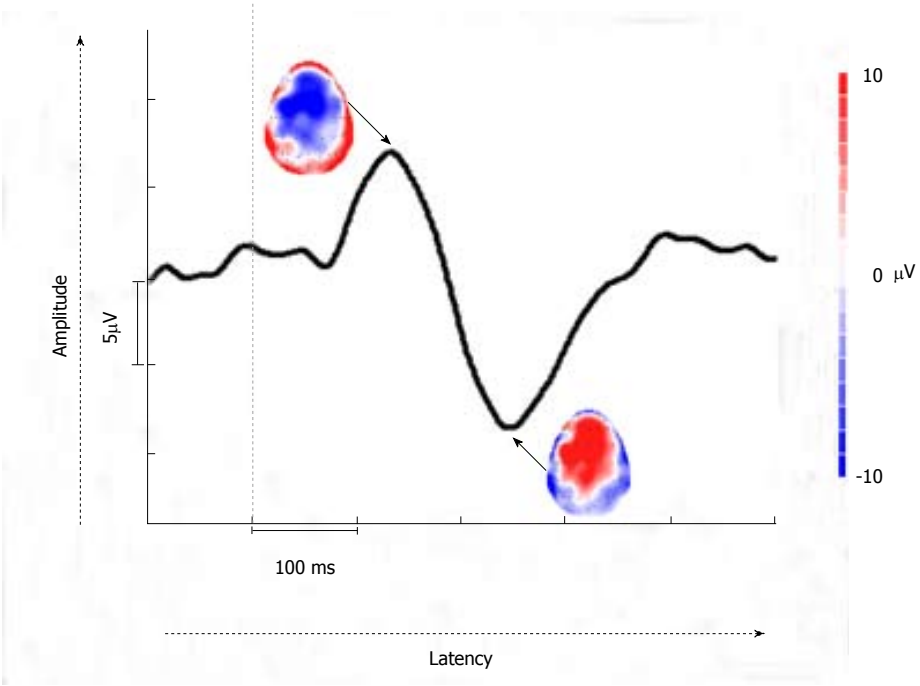

Figure 2 A typical visceral evoked potential at the central site on the scalp (Cz electrode). The two main peaks can be seen from the waveform. The two topographies at each of the peaks, representing the amplitude of evoked potentials over the entire scalp, are shown. Evoked potential waveforms and topographies can be used to analyze changes in amplitudes, latencies, and topographical distribution due to pain in chronic pancreatitis.

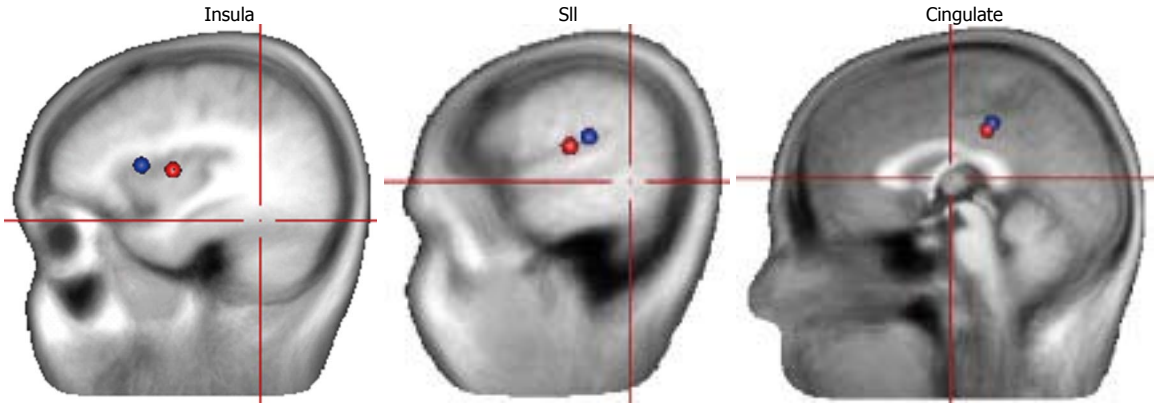

Figure 3 Analysis of underlying sources. This figure shows a typical 5-source model of visceral evoked potentials (bilateral insula, bilateral secondary somatosensory cortex, and cingulate). A posterior shift of the insula source can be seen[27]. SII: Secondary somatosensory cortex.

the CP patient group, likely reflecting reorganization of central pain pathways[9]. In a clinical study, where thirty-one patients with CP were randomly assigned to receive increasing doses of pregabalin or placebo for three consecutive weeks, no differences in rectal EP characteristics or their sources were seen, neither after pregabalin or placebo treatment[28]. However, since pregabalin was an effective drug significantly increasing patients' pain thresholds, the lack of changes in EP characteristics likely implies that visceral pain is mediated through subcortical mechanisms in patients with CP.

Somatic EPs

There is a lack of studies with somatic EPs in CP patients. However, recently CHEPs have received attention due to the stimulation being non-invasive and the relative selective activation of nociceptors. One study was done with CHEPs to investigate whether habituation was abnormal in CP patients[7]. The stimulation sites were the upper abdominal region (pancreatic "viscerotome") and right forearm (heterologous area). It was seen that during the repetitive stimuli, the CHEPs amplitudes increased in patients, although more prominently after stimulation of the upper abdominal region (25% as compared to 3% after arm stimulation), whereas in healthy controls, the amplitudes decreased during the repetitive stimuli by 20% (as expected). As the upper abdominal area shares spinal innervation with the pancreatic gland, these findings likely reflect abnormalities in cerebral pain processing distinctive of CP. Brain source analysis was done for these CHEPs data (unpublished) and a posterior shift of the operculum (representing

Table 1 Summary of findings of electroencephalography studies in chronic pancreatitis to date

Ref.	Methods	Results
Dimcevski *et al*[8], *Gastroenterology*, 2007	64-channel EPs; 12 HV and 10 CPP Electrical stimulation of the oesophagus, stomach, and duodenum Amplitudes, latencies, and brain sources of the EPs were analysed	Decreased latencies of the early EP components The bilateral insular sources localized more medial after stimulation of all 3 gut segments The cingulate source localized more posterior after stimulation of oesophagus
Drewes *et al*[26], *World J Gastroenterol*, 2008	62-channel EPs; 12 HV and 8 CPP Electrical stimulation of the oesophagus	Higher activity in theta band The main theta components oscillated with 4.4 Hz in the patients and 5.5 Hz in the controls
Olesen *et al*[27], *Pancreatology*, 2010	Topographic matching pursuit was used to extract the EEG information in the early brain activation after stimulation	The energy in the delta band was higher in healthy volunteers
	62-channel EPs; 14 HV and 24 CPP Electrical stimulation of the sigmoid Patients' daily pain experience was recorded in a pain diary Amplitudes, latencies, and brain sources of the EPs were analysed Correlation analysis was done between patients' pain scores and the changes in brain sources	EP latencies at frontal electrodes were prolonged The insular dipoles were localised more posterior The shift of insular dipole localisation was negatively correlated with the patients' clinical pain scores
Olesen *et al*[9], *Clin Gastroenterol Hepatol*, 2010	62-channel EPs; 15 HV and 25 CPP	Increased latency of P1
Olesen *et al*[21], *Eur J Gastroenterol Hepatol*, 2011	Electrical stimulation of the sigmoid Amplitudes and latencies of the EPs were analysed	Increased activity in delta, theta and alpha bands, and decreased beta band activity
	62-channel spontaneous EEG; 15 HV and 31 CPP Wavelet frequency analysis was used to retrieve amplitude strengths of the EEG The amplitude strengths were summarized in frequency bands with corresponding topographies	Differences in theta activity were located over centro-frontal brain regions, whereas differences in other frequency bands were located over frontal regions
Olesen *et al*[28], *Aliment Pharmacol Ther*, 2011	62-channel EPs 31 CPP randomly assigned to receive increasing doses of placebo or pregabalin over 3 wk Electrical stimulation of the sigmoid Amplitudes, latencies, and brain sources of the EPs were analysed	No differences in any of the EP characteristics between pregabalin and placebo groups
Olesen *et al*[7], *Eur J Pain*, 2013	Three sequences of 62-channel CHEPs; 15 HV and 15 CPP	During successive stimulation of the pancreatic area, N2/P2 amplitude increased 25% in CP patients, while it decreased 20% in healthy volunteers
	Upper abdominal region (pancreatic "viscerotome") and the right forearm (heterologous area) were stimulated. Habituation was calculated as the relative change in CHEPs amplitudes between the first and the third stimulation sequence	After stimulation of the forearm, N2/P2 amplitudes increased 3% in CP patients compared to a decrease of 20% in healthy volunteers

EPs: Evoked potentials; HV: Healthy volunteers; CP: Chronic pancreatitis; CPP: Chronic pancreatitis patients; EEG: Electroencephalography; CHEPs: Contact heat evoked potentials.

insula and secondary somatosensory cortex) source and an anterior shift of the cingulate source were observed following the stimulation of upper abdominal area. The operculum shift was positively correlated to the patient symptom score. No changes were seen in CP patients following stimulation of the arm. Since source position changes were only seen after stimulation of the area sharing spinal innervation with the pancreatic gland and these changes correlated to the patients' pain scores, the results are probably a reflection of maladaptive neuroplastic changes characteristic of CP.

CONCLUSIONS AND FUTURE PERSPECTIVES

The focus of pain treatment in CP has for many years concentrated on pathological findings in the pancreatic gland or extra pancreatic causes of pain. However, as we learn more about the mechanisms underlying pain in CP, it is clear that many patients will also benefit from treatments targeting central pain mechanisms[1]. A major obstacle to achieve successful treatment responses is to identify patients who will specifically benefit from these treatments (*i.e.*, patients with evidence of abnormal cen-

tral pain processing). Therefore, simple and objective diagnostic methods to identify abnormal central pain processing are highly desirable.

As discussed in this review, the electrophysiological research points to three cortical phenomena in CP: (1) thalamocortical dysrhythmia, as evident in increase of theta band activity in resting-state EEG and esophageal EPs; (2) cortical hyperexcitability as reflected in patients' lack of habituation to contact heat stimulation. The cortical hyperexcitability phenomenon could also be a consequence of thalamocortical dysrhythmia as happens in migraine[29]; and (3) reorganization within insular cortex following visceral and contact-heat EPs.

The electrophysiological methods reviewed in the present paper may be used as tools to unravel the origin of pain in patients with CP. Thereby patients with abnormalities in central pain processing can be identified prior to treatment assignment and thus the methods can assist clinicians when establishing treatment indications for pancreatitis pain in the individual patient (*e.g.*, invasive procedures *vs* medical treatment). However, further longitudinal clinical studies are needed to establish the value of the methods in the management of CP pain.

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MINIREVIEWS

Quality of healing of gastric ulcers: Natural products beyond acid suppression

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Abstract

Gastric ulcer is a chronic disease featured with unexpected complications, including bleeding, stenosis and perforation, as well as a high incidence of recurrence. Clinical treatments for gastric ulcer have allowed the rapid development of potent anti-ulcer drugs during the last several decades. Gastric ulcer healing is successful with conventional treatments including H_2-receptor antagonists, and proton pump inhibitors (PPIs) have been essential for ulcer healing and prevention of complications. Additionally, *Helicobacter pylori* eradication therapy is effective in reducing ulcer recurrence and leads to physiological changes in the gastric mucosa which affect the ulcer healing process. However, in spite of these advancements, some patients have suffered from recurrence or intractability in spite of continuous anti-ulcer therapy. A new concept of the quality of ulcer healing (QOUH) was initiated that considers the reconstruction of the mucosal structure and its function for preventing ulcer recurrence. Although several gastroprotection provided these achievements of the QOUH, which PPI or other acid suppressants did not accomplish, we found that gastroprotection that originated from natural products, such as a newer formulation from either *Artemisia* or S-allyl cysteine from garlic, were very effective in the QOUH, as well as improving clinical symptoms with fewer side effects. In this review, we will introduce the importance of the QOUH in ulcer healing and the achievements from natural products.

Key words: Quality of ulcer healing; Natural products; Gastric ulcers; Acid suppression; S-allyl cysteine; *Artemisia* isopropanol extract

Core tip: Clinical treatments for gastric ulcer have allowed rapid development of potent anti-ulcer drugs, such as H_2-receptor antagonists or proton pump inhibitors, which has been essential for ulcer healing and the prevention of complications. However, many patients have still suffered from recurrence or intractability in spite of continuous anti-ulcer therapy. The concept of the quality of ulcer healing is that we should consider the reconstruction of the mucosal structure and its function for preventing ulcer recurrence. In this review, we will introduce the importance of the quality of ulcer healing and the achievements from natural products.

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INTRODUCTION

Gastric ulcers affect many people worldwide and are characterized by the presence of a deep necrotic lesion involving the entire mucosal thickness and the muscularis mucosae[1,2]. Gastric ulcer is a chronic disease featured with repeated healing and recurrence in the original location or elsewhere throughout the patient's lifetime. Basically, its development is a result from an imbalance between mucosal defensive mechanisms, including mucus, bicarbonate, prostaglandins (PGs) and mucosal blood flow, and damaging factors, including acid and pepsin, in the luminal surface of the gastric mucosa[2]. Among these factors, the two major damaging causes implicated in gastric ulcer and ulcer recurrence are infection with *Helicobacter pylori* (*H. pylori*) and long-term use of nonsteroidal anti-inflammatory drugs (NSAIDs)[3,4]. Toxins of *H. pylori* and NSAIDs disturb the ulcer healing mechanism by inhibiting epithelial cell proliferation, migration and angiogenesis, and by blocking growth factor triggered signaling pathways[5-7]. Gastric ulcers respond to conventional treatment, including H_2-RA, acid suppressant drugs and antibiotic drugs for eradication of *H. pylori,* as well as the withdrawal of NSAIDs. However, several studies have shown that, in the ulcer healing process, acid inhibition or *H. pylori* eradication is insufficient for complete ulcer healing since the decrease of PGs and the increase of oxygen free radical leads to a varying quality of ulcer healing and is intimately associated with ulcer recurrence[8,9].

In 1993, Arakawa *et al*[10] proposed the concept and definition of the quality of ulcer healing (QOUH) based on histological maturity of regenerated tissue in the ulcer area, for which the additional intervention might be a prerequisite, including PGs, radical scavengers and the way of spurting regeneration, as well as the removal of inflammation. Since several studies have reported that a low recurrence rate is intimately associated with the achievement of the QOUH, efforts to achieve the QOUH might impose the ideal healing of an ulcer as well as functional restoration. The QOUH is achieved by several kinds of gastroprotective drugs, including rebamipide, sucralfate, misoprostol, ecabet sodium, sofalcone and antacids[6,11-15]. Rebamipide is one of the gastroprotective drugs able to intervene effectively in the process of ulcer healing and effectively improve the QOUH in the chronic acetic acid ulcer model. The combination of omeprazole and rebamipide accelerated the quality of ulcer healing through an increasing level of prostaglandin E2 (PGE_2) and a decreasing level of Interleukin-8 (IL-8) and malondialdehyde (MDA) in the gastric mucosa, but not omeprazole alone[6]. In addition, rebamipide inhibited *H. pylori* CagA-induced effects on gastric epithelial cells, including morphological changes associated with ZO-1, IL-8 production and nuclear factor kappa B (NF-κB) activation in gastric epithelial cells[16].

The antacid talcid activates epidermal growth factor (EGF) and EGF-R expression in normal and ulcerated gastric mucosa. EGF and EGF-R are crucial for cell proliferation, migration, re-epithelialization and gland reconstruction within the scar. In addition, antacids, especially hydrotalcite, cause activation of prostaglandin synthesis; binding to and inactivation of pepsin, lysolecithin, bile acids and *H. pylori* toxins; activation of heat shock proteins; and activation of the genes encoding EGF, bFGF and their receptors[17]. Several diverse studies revealed the significance of the gastric defense system to overcome these limitations of acid suppressant treatment with ecabet sodium, *Artemisia* extracts and sucralfate.

Recently, we identified that natural product substances have an increasing interest because of fewer side effects, low cost and a potential source for anti-ulcer treatment through improving the QOUH and preventing ulcer recurrence. In this review, we discuss numerous natural products, including isopropanol, ethanol extracts of *Artemisia,* extracts from garlic and a special licorice extract, and their secondary metabolites with potential gastroprotection to achieve the QOUH and resistance to ulcer recurrence.

MECHANISMS OF GASTRIC ULCEROGENESIS AND QOUH

Mechanism of gastric ulcerogenesis

Gastric ulcers are a deep defect in the gastric wall involving the mucosal thickness and the muscularis mucosae. A gastric ulcer results from tissue necrosis induced by various conditions, such as aspirin, indomethacin, bile acids, alcohol intake, ischemia, stress, aging and *H. pylori* infection, the so called excess increment of damaging factors far exceeding the defense capacity. These conditions are known to disturb the mechanisms of gastric mucosal defense and develop characteristic morphological, ultrastructural and functional changes. When gastric mucosa is exposed to damaging agents, it encompasses the disruption of the unstirred mucus/bicarbonate/phospholipid layer, exfoliation of the surface epithelium with loss of its barrier and the deeper gastric mucosal layers, including microvascular endothelial cells, progenitor, parietal and chief cells. When the capillary endothelium was damaged, it led to microvascular stasis with cessation of oxygen and nutrient delivery and hypoxia. Microvascular damage occurs early during mucosal injury, leading to hypoxia and necrosis of glandular cells and thus adding an ischemic component to the direct toxic injury of cells. Vasoconstriction events produced by release of vasoactive and proinflammatory mediators from damaged mast cells, macrophages and endothelial cells further impair the mucosal microcirculation and ultimately result in mucosal necrosis in the form of ulcers[2,17]

Gastric ulcer healing

Ulcer healing is an orchestrated process of filling the mucosal defect with epithelial and connective tissue cells,

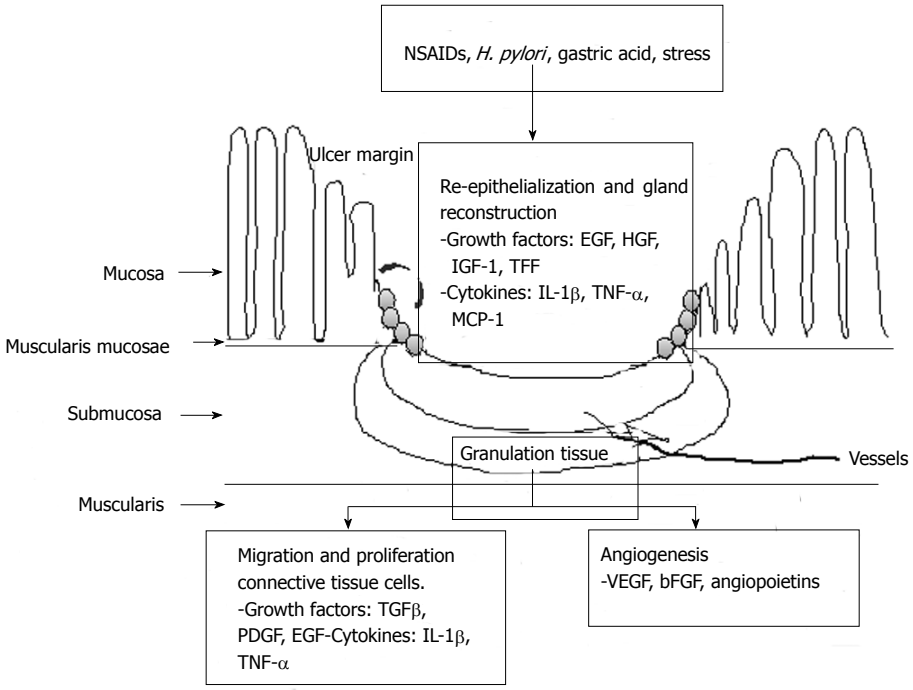

Figure 1 Schematic representation of intracellular pathway on ulcer healing mechanism. SAC: Artemisia or S-allyl cysteine; PPI: Proton pump inhibitor; NSAIDs: Nonsteroidal anti-inflammatory drugs; TNF-α: Tumor necrosis factor-α; MCP-1: Monocyte chemotactic protein-1; EGF: Epidermal growth factor; HGF: Hepatocyte growth factor; VEGF: Vascular endothelial growth factor; IL-1β: Interleukin-1β; IGF-1: Insulin like growth factor-1.

including cell proliferation, migration, differentiation, regeneration, active angiogenesis and extracellular matrix deposition, leading to scar formation. The structure of a gastric ulcer consists of an ulcer margin, which is epithelial tissue formed by adjacent non-necrotic mucosa, and the granulation tissue which is connective tissue[2], as shown in Figure 1. At the ulcer margin, epithelial cells proliferate and migrate onto the granulation tissue to cover (re-epithelialize) the ulcer and initiate reconstruction of the glands within the ulcer scar.

The processes of re-epithelialization and gland reconstruction are controlled by growth factors, including EGF, hepatocyte growth factor and insulin like growth factor-1 (IGF-1) , as well as trefoil factors, including pS2, Trefoil factor family Ⅱ (TFF Ⅱ) and Intestinal trefoil factor, prostaglandins generated through activated cyclooxygenase-2 (COX-2), and other cytokines produced locally by regenerating cells in an orderly and integrated manner. These factors, mainly EGF and prostaglandins, trigger cell proliferation *via* signal transduction pathways involving both direct activation and transactivation of the EGF receptor. Granulation tissue develops at the ulcer base. It consists of fibroblasts, macrophages and proliferating endothelial cells forming microvessels under the control of angiogenic growth factors, including vascular endothelial growth factor (VEGF), bFGF and angiopoietins. The angiogenesis is essential for the restoration of the blood microcirculation in the mucosa and is thus crucial for oxygen and nutrient supply.

The major mechanism underlying the activation of angiogenic growth factors and expression of their receptors is hypoxia, which activates the transcription factor, hypoxia-inducible factor 1 (HIF-1α). HIF-1α, in turn, up-regulates VEGF transcriptional expression and thus increases the local production of VEGF essential for angiogenesis. The final outcome of the healing process reflects a dynamic interaction between the epithelial component from the "healing" zone at the ulcer margin and the connective tissue component (including microvessels) originating from the granulation tissue and from bone marrow derived stem cells attracted to the site of injury[2,17].

Gastric ulcer recurrence

Numerous neutrophils and macrophages persist in and beneath the regenerated epithelium, even after ulcer healing, the basis for ulcer recurrence. This persistent chronic inflammation may have a key role in causing future ulcer recurrence. Watanabe *et al*[18] demonstrated that inflammatory cytokines, IL-1β and tumor necrosis factor (TNF)-α administered systemically in rats with a macroscopically healed gastric ulcer cause ulcer recurrence at the site of the previous ulcer. This model of gastric ulcer recurrence has found increased expression of adhesion molecules, intercellular adhesion molecule (ICAM-1) in endothelial cells and leukocytic β2 integrins, lymphocyte function-associated antigen and Mac-1 in leukocytes and cytokines, IL-1β and TNF-α, and chemokine, monocyte chemotactic protein (MCP)-1. This increase occurred in the regenerated tissue of the healed ulcer site, the ulcer scar, 12 h after injection of an inflammatory cytokine and was followed by massive infiltration of macrophages and neutrophils, ultimately resulting in ulcer recurrence. Anti-neutrophil antiserum prevents ulcer recurrence in this

model, suggesting that neutrophils producing noxious protease and active oxygen species are the final mediator of tissue injury. These molecules regulate migration of neutrophils from arterioles into the interstitial space. An antibody against MCP-1 prevents gastric ulcer recurrence in this model, suggesting that the overexpression of MCP-1 in resident macrophages accumulated in the interstitial space of the ulcer scar is a first step in the mechanism of ulcer recurrence because neutrophils and macrophages infiltrate the interstitial space of the ulcer scar only after overexpression of MCP-1[19].

FACTORS AFFECTING THE "QOUH"

Tarnawski *et al*[1] and Arakawa *et al*[10] first proposed the concept of the QOUH. Since the QOUH is defined as histological maturity of regenerated tissue replaced at an ulcer site, evaluation of the QOUH should be done to assess functional and endoscopic maturity in addition to histological maturity[10]. High QOUH is ideal ulcer healing featuring a fine granular ulcer scar, high functional restoration of mucosa and granulation tissue, and the resistance to recurrence[10]. In a clinical study, QOUH was assessed by chromoendoscopy. The recurrence is minimal from a high quality ulcer scar (flat scar), while it frequently occurs from a poor one (nodular scar)[20]. Accumulation of macrophages and expression of cytokines are much more prominent in a poor quality scar than a high quality scar. The acetic acid ulcer model in rat has been developed[21] and is used as a standard model for screening new anti-ulcer drugs. This model closely resembles human ulcers in terms of both pathological features and healing mechanisms and the ulcer responds well to various anti-ulcer drugs[22].

NSAIDs are a major cause of ulcer complications and deaths worldwide. Arakawa *et al*[23] demonstrated that indomethacin administered during the initial period of acetic acid-induced gastric ulcer healing affects future ulcer recurrence. Cumulative ulcer recurrence rate was significantly higher in rats initially treated with indomethacin than in controls. Increased polymorphonuclear neutrophil (PMN) infiltration was the major histological abnormality persisting after cessation of indomethacin. Therefore, the administration of indomethacin during the initial period of ulcer healing promoted persistent PMN infiltration and increased ulcer recurrence rates, possibly *via* a prostaglandin-dependent mechanism[23]. On the other hand, Wang *et al*[4] demonstrated the effects of aspirin on ulcer recurrence and healing quality. Aspirin was administrated by gavage from day 25 to 54 after ulcer induction by an acetic acid-induced ulcer in rat. The gastric juice volume was significantly increased in the aspirin group compared with those of fasting or saline control groups, while the pH, mucus, mucosal blood flow and PGE_2 were significantly decreased in the aspirin treated rats compared with those of the other two groups. COX-2 was significantly augmented in the aspirin group compared with the others. The QOUH in the aspirin group was poorer than that of the fasting or saline control groups. The imbalance between protective and aggressive factors resulted in poor QOUH and ulcer recurrence. Therefore, improvement of the QOUH may contribute to decrease the incidence of ulcer recurrence and the development of a new anti-ulcer treatment.

Prostaglandins

PGs play a critical role in the regulation of gastric acid secretion and maintaining gastric mucosal integrity. The achievement of QOUH is thought to be PG-dependent. Exogenous PGs could reverse events involved in ulcer recurrence, inflammatory response, retarded ulcer healing and defective angiogenesis[4,13,16].

Vascular endothelial growth factor and angiopoietin 1

Angiogenesis is crucial for gastric ulcer healing which is stimulated by VEGF and angiopoietin-1. Joes *et al*[24] found that local gene therapy with VEGF and Ang1 into the ulcer base, with limited duration of target gene expression, significantly increased neovascularization and accelerated ulcer healing in rats. Furthermore, co-injection of both plasmids encoding rhVEGF 165 and rhAng1 resulted in formation of more mature vessels and a more complete restoration of gastric glandular structures within the ulcer scar, reflecting better QOUH.

Heat shock protein

Heat shock protein (HSP) acts as a molecular chaperone and exhibits various functions, including protection against apoptosis, protease inhibition and cross-linkage to other proteins. The induction of HSPs is important for protection against apoptosis, protease inhibition, refolding and activity of partially denatured proteins; other reports indicate that their beneficial effect may be especially important for the later phase of regeneration when oxidative stress caused by infiltrating inflammatory cells may oppose tissue repair, signifying that HSP27 induction might be one of the mechanisms that lower recurrence of gastric ulcer after IL-1β in gastroprotective administration[25].

Inflammatory cells and cytokines

The number of macrophages infiltrating scar tissue is five times higher than neutrophils in a non-flat scar, suggesting that these macrophages may play a key role in the pathophysiology of the QOUH and thus future ulcer recurrence. These macrophages produce increased amounts of IL-1β, TNF-α and MCP-1. The proinflammatory cytokines, IL-1β and TNF-α, further activate and stimulate macrophages, thus constituting a self-perpetuating circuit. The increased stimulation of production of these cytokines induced by NSAIDs, stress or *H. pylori* may cause these macrophages to increase cytokine production and/or release, leading in turn to attraction and accumulation of neutrophils. Neutrophils release proteases and active oxygen species, damage the scar tissue and induce ulcer

recurrence[19].

ANTI-ULCER TREAMENT TO ACHIEVE THE QOUH

Clinical treatments for gastric ulcer have allowed rapid development of anti-ulcer drugs and gastric ulcer healing is successful with conventional treatments, including H_2-RA, PPI and the eradication of *H. pylori* infection. However, numerous studies have shown that during the ulcer healing process, either acid inhibition or *H. pylori* eradication is not enough to prevent relapse as well as complications. These treatments were found to be insufficient to remove or clear accumulated inflammation beneath the ulcer scar, decreased PGs and excessive oxidative stress, all of which affected the QOUH and ulcer recurrence. Therefore, a new concept of the QOUH was initiated to consider the reconstruction of the mucosal structure and ideal functional restoration, as well as clearing gastric inflammation to prevent ulcer recurrence.

More gastric ulcer recurrence develops after completion of conventional treatment in patients whose ulcers heal with an H_2-RA than in patients treated with other drugs. Arakawa *et al*[26] found that inhibition of acid secretion significantly accelerates ulcer healing; however, acid suppressors used alone cannot improve the quality of healing. Combination treatment with both sucralfate (sucrose aluminum sulfate) and omeprazole treatment can improve the QOUH. The stimulating actions of sucralfate on EGF and angiogenesis may be the basis for improving the QOUH[27,28]. Several kinds of gastroprotective drugs are available in clinics, including rebamipide, ecabat sodium and *Artemisia* extracts, that achieves QOUH and prevents ulcer recurrence. For instance, rebamipide is a gastroprotective drug that induces mucosal prostaglandin generation, accelerates ulcer healing and reduces relapse of the acetic acid ulcer model in rats. Arakawa *et al*[29] reported that administration of rebamipide during the initial period of acetic acid-induced gastric ulcer affected healing and future ulcer relapse. The ulcer healing rate was higher in rats given rebamipide alone than in those given rebamipide and cimetidine during and after administration, but not in rats given cimetidine alone. The relapse rate was significantly lower in rats initially given rebamipide alone or those given rebamipide and cimetidine than in rats initially given cimetidine alone. Therefore, rebamipide is beneficial for improving the QOUH and reduction of future ulcer relapse[29]. Additionally, rebamipide as well as omeprazole and the combination regimen may improve the QOUH through increasing the level of PGE_2 and decreasing the levels of IL-8 and MDA in gastric mucosa. This may potentially result in reduced recurrence of the ulcer. Moreover, the combination regimen was identified as having more anti-ulcer effects than monotherapy[6].

Eradication of *H. pylori* leads to healing of acute inflammation of the gastric mucosa, followed by changes in the gastric environment such as gastric acid secretion. Therefore, gastroprotective drugs such as repamipide should be given for post-eradication physiological changes in the gastric mucosa to promote ulcer healing and prevent ulcer recurrence[30]. Kato *et al*[31] showed that the administration of rebamipide with teprenone is a combination for dual therapy for *H. pylori* eradication. A total of 102 *H. pylori*-positive gastric ulcer patients were assigned at random to two groups. In addition to dual therapy (amoxicillin 500 mg thrice daily and lansoprazole 30 mg every morning for two weeks), one group received rebamipide 100 mg thrice daily for eight weeks, while the other group received teprenone 50 mg thrice daily for eight weeks. The ulcer healing rate was 85.7% in the rebamipide group and 79.5% in the teprenone group. The eradication rate was 68.4% in the rebamipide group and 47.7% in the teprenone groups. These findings suggest that the efficacy of dual therapy may be increased by the administration of rebamipide. Lafutidine is one of anti-ulcer drugs with antisecretory and gastroprotective activities[32], mediated by capsaicin-sensitive sensory neurons (CSSN). Lafutidine (30 mg/kg) significantly accelerated the ulcer healing and the recurrence rate was much lower than that for the vehicular control. In CSSN-desensitized rats, lafutidine also accelerated the ulcer healing significantly, but the low recurrence rate shown in normal rats was counteracted. The recurrence rate of the combination of famotidine and teprenone (100 mg/kg) was lower than that of famotidine alone. Therefore, the low recurrence rate of ulcers after lafutidine treatment in rats seems to depend on the gastroprotective mechanisms involving CSSN[33].

GASTROPROTECTIVE EFFECT OF NATURAL PRODUCTS TO ACHIEVE QOUH

Natural products and their compounds are highly effective in anti-ulcer treatment, possessing a gastroprotective action and being gentle on the body's systems without any side effects. While many synthetic and prescription drugs provide symptomatic relief, they also have harsh side effects. Our previous studies have shown that gastroprotective actions from *Artemisia* ethanol extract treatment was quite efficient in accelerating the ulcer healing at the early phase of ulcer healing and hindering the recurrence of gastric ulcer after complete ulcer healing, whereas an acid suppressant was somewhat inferior in these aspects of ulcer healing. The achievement of ideal QOUH can only be accomplished with intervention of the enhancement of gastric defense systems. The molecular basis for the QOUH achievement with *Artemisia* treatment were efficient remodeling of regenerated gastric mucosa, intervention of several growth factors, abundant gastric mucins, including trefoil proteins like trefoil peptide (pS2/TFF1), and significant suppression of inflammatory cytokines like IL-2, TNF-α, COX-2 and nitrosative stress. Also, mechanisms of *Artemisia* extract showing resistance to ulcer recurrence were excellent remodeling activity, enrichments of molecular chaperones like HSP27, as

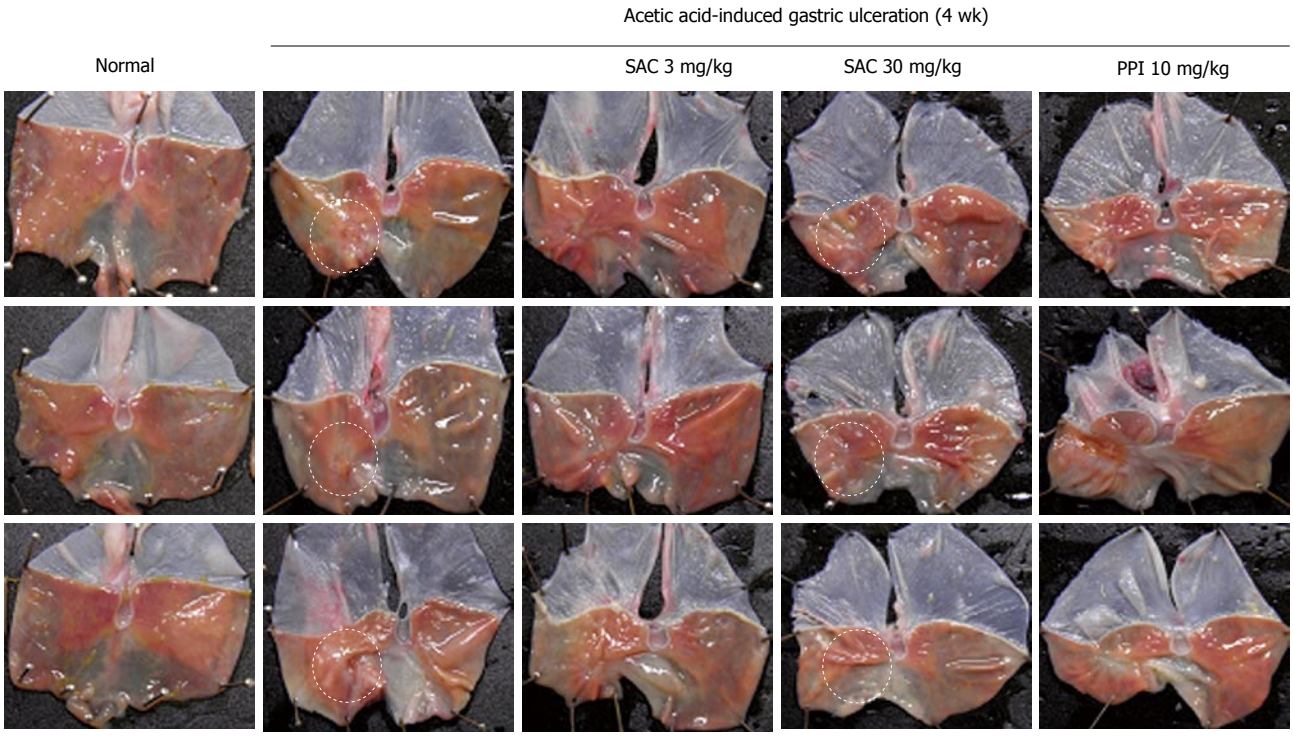

Figure 2 Gross features of gastric ulcer stage assessed at 4 wk after acetic acid serosa injection according to group. SAC: *Artemisia* or S-allyl cysteine; PPI: Proton pump inhibitor.

well as significant decreases in inflammatory cytokines like COX-2, IL-2 and TNF-α, and quenching nitrosative stress.

In our recent study, *S*-allyl cysteine (SAC), an organic compound that is a natural constituent of fresh garlic, has been shown to exert anti-inflammatory and anti-oxidative effects by numerous studies. SAC significantly increased the total antioxidant concentration and decreased levels of MDA, a lipid peroxidation marker in water immersion restraint stress-induced gastritis *in vivo*, suggesting that SAC prevents stress-induced gastritis through enhancing antioxidative activity. SAC significantly inhibited TNF-α-induced pro-inflammatory signaling, including COX-2, inducible nitric oxide synthase and cytosolic phospholipase A. Moreover, SAC suppressed TNF-α-induced phosphorylation and subsequent degradation of IκBα by preventing IKKβ activation, thereby inhibiting TNF-α-induced nuclear translocation of NF-κB p65. Our results suggest that SAC can be a gastroprotective agent against stress-induced gastric mucosal damage by potentiating antioxidative activity and suppressing NF-κB activation and subsequent proinflammatory cascades. Also, in the acetic acid ulcer model, the healing rate was higher in rats treated with SAC 30 mg/kg than with PPI administration, as shown in Figure 2. Additionally, we have added more evidence that isopropanol extracts of *Artemisia* possessed higher antioxidative and anti-inflammatory actions (data not shown), inferring a higher achievement of QOUH in gastric ulcer models. Therefore, although acid suppressant is the gold standard treatment for a gastric ulcer, the antisecretory agents alone were not sufficient for reaching the QOUH, necessitating the combined use of antisecretory and gastroprotective agents for anti-ulcer treatment.

Other studies have shown the gastroprotective effect of *Pongamia pinnata* root flavonoids (PRF) that significantly inhibited ulcerative formation and increased the EGF and TGF-α expression of para-ulcer mucosa tissue and improved the EGF contents in blood serum, which might be one of possible mechanisms by which PRF improves the QOUH[34]. The stem bark of *Lafoensia pacari* is a traditional medicine in Brazil widely used for the treatment of gastroduodenal ulcers. The gastroprotective and ulcer healing of methanol extract of *Lafoensia pacari* (MELP) was evaluated using ethanol, indomethacin, cold-restraint stress-induced and acetic acid ulcer models in experimental animals. This study has shown that MELP possesses preventive and curative effects against gastric ulcers. These effects are partly dependent on its antioxidant, antisecretory properties and inhibition of pro-inflammatory cytokines and independent of gastric motility and mucus secretion[35]. The ethanolic extract (EET) of roots from *Arctium lappa* (bardana) has shown that it accelerates the healing of acetic acid-induced gastric ulcers in rat. Oral administration of EET reduced the gastric lesion area and promoted regeneration of the gastric mucosa. EET also restored the superoxide dismutase activity, prevented the reduction of glutathione levels, reduced lipid hydroperoxide levels, inhibited the myeloperoxidase activity and reduced the microvascular permeability. In addition, EET reduced the free radical generation and increased scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH)

free radicals *in vitro*. Furthermore, intraduodenal EET decreased the volume and acidity of gastric secretion. Therefore, the gastroprotective effect of EET was mediated by the decrease in the volume and total acidity of gastric secretion, cell proliferation, reduction of the inflammatory process and antioxidant-dependent mechanisms[36].

Pimple *et al*[37] investigated the gastroprotective effect of *Luffa acutangula* methanolic extract (LAM) and aqueous extract (LAW) on type II diabetes rats. LAM significantly increased mucosal glycoprotein and antioxidant enzyme levels in the gastric mucosa of diabetic rats, more than LAW. LAM was efficient in reversing the delayed healing of gastric ulcers in diabetic rats close to the normal level. LAM exhibited a better ulcer healing effect than glibenclamide and LAW, because of its antihyperglycemic and mucosal defensive actions. *Morus alba* is a well-known Chinese herb traditionally used for the prevention and treatment of several diseases as this plant possesses antidiabetic, hypolipidemic, antimicrobial, antioxidant and anti-ulcer activities. Five new compounds were isolated, one a new steroid named albo steroid. This new compound exhibits significant anti-ulcer activity in pylorus-ligation and ethanol-induced ulcer models. Furthermore, this compound showed significant dose-dependent reversal of ethanol-diminished activity in antioxidant enzymes, such as superoxide dismutase, catalase, glutathione peroxidase and glutathione, and reduced the ethanol-elevated levels of glutathione reductase and lipid peroxidation[38]. Finally, VSL#3 is a probiotic preparation containing a mixture of eight bacterial species, including *Lactobacilli*, *Bifidobacteria* and *Streptococcus* species which has been shown to be highly effective in accelerated gastric ulcer healing by stimulating the expression and secretion of angiogenesis promoting growth factors, primarily VEGF. The expression and protein production of VEGF was significantly increased on day 7 in the ulcerated tissues of VSL#3 treated animals. In addition, animals treated with VEGF neutralizing antibody significantly delayed gastric ulcer healing in VSL#3 treated animals[39].

CONCLUSION

We cannot ignore the principal role of acid suppressant in the treatment of gastric ulcers. Based on the experimental documentation, we suggest that the combined use of acid suppressants and gastroprotection should be considered to improve the quality of ulcer healing, facilitating rapid symptom relief, accelerated healing and resistance to ulcer recurrence, as well as complete functional restoration. In conclusion, our novel finding is that gastroprotective treatments are not merely supplementary in the treatment of gastric diseases, otherwise signifying that gastroprotection might be essential and a prerequisite for better healing, for which phytoceuticals or phytochemicals can be ideal candidates, supported with safety and effectiveness.

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MINIREVIEWS

Microscopic colitis: Common cause of unexplained nonbloody diarrhea

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Abstract

Microscopic colitis (MC) is characterized by chronic, watery, secretory diarrhea, with a normal or near normal gross appearance of the colonic mucosa. Biopsy is diagnostic and usually reveals either lymphocytic colitis or collagenous colitis. The symptoms of collagenous colitis appear most commonly in the sixth decade. Patients report watery, nonbloody diarrhea of a chronic, intermittent or chronic recurrent course. With collagenous colitis, the major microscopic characteristic is a thickened collagen layer beneath the colonic mucosa, and with lymphocytic colitis, an increased number of intraepithelial lymphocytes. Histological workup can confirm a diagnosis of MC and distinguish the two distinct histological forms, namely, collagenous and lymphocytic colitis. Presently, both forms are diagnosed and treated in the same way; thus, the description of the two forms is not of clinical value although this may change in the future. Since microscopic colitis was first described in 1976 and only recently recognized as a common cause of diarrhea, many practicing physicians may not be aware of this entity. In this review, we outline the epidemiology, risk factors associated with MC, its etiopathogenesis, the approach to diagnosis and the management of these individuals.

Key words: Collagenous colitis; Lymphocytic colitis; Microscopic colitis; Intraepithelial lymphocytes; Thickened collagen band; Diarrhea-predominant irritable bowel syndrome

Core tip: The gastroenterologist and gastrointestinal pathologist should keep this common condition in mind as a cause of unexplained nonbloody diarrhea with normal mucosal appearance on colonoscopy to avoid misdiagnosis that may affect treatment of patients. Referral of patients with pathogen negative chronic diarrhea to medical centers that have facilities for colonoscopy and biopsy is vital in the developing world.

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INTRODUCTION

Microscopic colitis is regarded as one of the common causes of chronic watery diarrhea. The incidence rate for collagenous colitis is 0.8/100000-6.2/100000. Many cases have been reported in western countries and in Asian countries like India. Lindstrom and Freeman described the term collagenous colitis concurrently in 1976. Microscopic colitis (MC) refers to two medical conditions which cause diarrhea: collagenous colitis and lymphocytic colitis. The following triad of clinicopathological features characterizes both conditions: (1) chronic watery nonbloody diarrhea; (2) normal mucosal appearance on colonoscopy; and (3) characteristic histopathology.

Patients are characteristically, although not exclusive-

ly, middle-aged females. They present with a long history of watery nonbloody diarrhea which may be profuse. There is a strong association between autoimmune diseases, for example arthritis, Sjorgen's syndrome and celiac disease, and microscopic colitis. There are reports of associations with multiple drugs, especially non-steroidal anti-inflammatory drugs. Colonoscopy is normal or near normal. The changes are often patchy, so multiple colonic biopsies must be taken in order to make a correct histological diagnosis[1-3]. A full colonoscopy is required as an examination limited to the rectum will miss cases of MC.

EPIDEMIOLOGY

The true incidence of MC is not known. The disease has been increasingly diagnosed over the past 20 years but is still uncommon. A recently published population-based study found the incidence of microscopic colitis to increase significantly from 1.1 per 100000 persons in the late 1980s to 19.6 per 100000 persons by the end of 2001. More recent epidemiological studies done in this century confirmed these high incidence numbers, showing that actual incidence and prevalence numbers are higher than initially thought and are still able to show rising incidences, although the rise is far less pronounced than before. Most recent north American studies show incidence rates of 7.1 per 100000 person-years for collagenous colitis and 12.6 per 100000 person-years for lymphocytic colitis[4].

Morbidity is limited to the consequences of diarrhea, including metabolic abnormalities such as hypokalemia and dehydration, weight loss and fatigue. This is not considered a life-threatening condition; however, profuse watery diarrhea may lead to severe dehydration and electrolyte abnormalities requiring intensive resuscitation.

Lymphocytic colitis affects similar numbers of men and women, while collagenous colitis is up to 20 times more frequent in women than in men[5].

Both conditions are observed most commonly in people over the age of 40 years, with peak incidence in the sixth and seventh decades of life, and the incidence of both conditions increases with age. Isolated cases have been reported in younger populations, including children[5-8].

ETIOPATHOGENESIS

The etiology of MC is most likely multifactorial with a mucosal inflammatory response to yet not specified noxious luminal agent occurring in a predisposed host. The noxious luminal agent may be a single one or multiple ones summing up to an individual threshold. Technically, MC is an inflammatory bowel disease (IBD) and the disease shares a number of etiological aspects with the so-called classical inflammatory bowel diseases like Crohn's disease and ulcerative colitis. Among the possible predisposing and/or contributing factors for microscopic colitis, genetic factors and intraluminal noxious factors are best studied. Although a limited number of familial clusters of microscopic colitis have been reported, there is only minimal evidence of a genetic component within the etiology of MC. All reported so-called family clusters are very small and comprise of a maximum of two reported family members. In contrast, there is evidence of a predisposition of sensitivity to gastrointestinal inflammatory insults in patients with microscopic colitis since up to 12% of patients with MC have a family history of celiac disease or even inflammatory bowel disease[8]. The meaning of the association between Human Leukocyte antigen (HLA-DQ2, DQ1, DQ3) and microscopic colitis and the high prevalence of a tumor necrosis factor (*TNFα*) gene polymorphisms in patients with microscopic colitis deserves further attention as it may lead to a discovery of a hereditary component of microscopic colitis of presently unknown penetration[9]. Furthermore, metalloproteinase-9 gene variations have been reported to be associated with collagenous colitis[10]. However, the meaning of all the presently reported genetic associations is poorly understood and the respective research is presently not driven by hypotheses, rather than by incidental observations or genetic screening. Very strong evidence exists for an autoimmune basis to the development of both collagenous and lymphocytic colitis. The association of MC with autoimmune-based disorders such as celiac, thyroid disease and rheumatoid arthritis, as well as the female preponderance, supports the notion that both forms of MC have a strong association with autoimmune diseases and may well be an autoimmune disorder themselves. However, to date, no specific autoantibody has been identified as being diagnostic for or being associated with collagenous or lymphocytic colitis[11]. It is known that MC can be found together with various autoantibodies and phenotypes, like human leukocyte antigen (HLA)-DR3 phenotype, although these associations are not strong enough to be regarded as being diagnostically relevant or useful, nor do we know what these associations mean. A case series of 4 patients is described in which subjects developed classic symptoms of lymphocytic-collagenous colitis with typical mucosal histopathology during treatment with omeprazole/esomeprazole (proton pump inhibitors)[12]. Symptoms promptly stopped and mucosal biopsies returned to normal with drug withdrawal. Disease quickly recurred in 2 patients who were re-exposed to the drugs, one with biopsy documented recurrent collagenous colitis. Luminal factors of whatever kind seem to play an important role in the pathogenesis of microscopic colitis. Numerous drugs were reported to have a high or at least intermediate probability of causality in microscopic colitis[13]. Other luminal factors like infectious or even toxic agents are supported by studies that found either onset of MC following a gastrointestinal infection or improvement of symptoms with the initiation of antibiotics in the context of a proven or suspected gastrointestinal infection Yesinia species[14], *Clostridium difficile*[15] and Campylobacter species[16] were suggested in published case reports to cause MC; although, interpreting these observations in the context of

current knowledge, it is most likely that these cases are of sporadic nature. In some small retrospective case series, bile acid malabsorption was found in up to 60% of patients with lymphocytic and up to 44% of patients with collagenous colitis, supporting the notion that MC may at least in some patients be caused by bile acid malabsorption. Whether bile acid malabsorption is causative or not remains questionable, as later studies were unable to confirm these observations[17]. Still, this may direct therapeutic decisions and especially in patients with a cholecystectomy, a bile acid directed treatment should be considered. Basic science is still in its infancy when it comes to studying microscopic colitis and possible causes, drivers, mechanisms or even pathophysiological models. A recent bench side study employing sigmoid tissues from patients with collagenous colitis and lymphocytic colitis was able to identify that sodium transport and epithelial barrier function are disturbed in patients with microscopic colitis[18]. Unfortunately, it remains unclear whether these reported changes are of causal nature, of transient nature, or a consequence of the underlying microscopic colitis. Even although these descriptive studies at least initiate a scientific discussion on what may be mechanisms underlying or involved in the development and the resolution of microscopic colitis, these small mechanistic studies have to be carefully taken since such studies are highly artificial in the techniques they use and therefore the results are most likely influenced not only by numerous circumstances like laboratory procedures and protocols but also by patient drug use, age and even nutritional status. Thus, such information has to be considered as hypotheses generating information that hopefully guides future prospective studies that help us to understand the mechanisms involved in the pathogenesis, maintenance and resolution of microscopic colitis and symptoms associated with the histological changes.

Using molecular techniques, it was reported that in patients with MC, increased interferon-γ, TNF-α and IL-1β levels suggest a Th1 cytokine profile being involved in the inflammatory process[18]. The differences in mucosal lymphocyte subsets seen in patients with collagenous and lymphocytic colitis[19] is not fully understood presently. This information may help us to understand the inflammatory mechanisms involved and may be useful for future therapeutic approaches. Environmental factors may play a crucial role in the etiology of MC, although other than cigarette smoking, presently no other factors are confirmed. For both collagenous and lymphocytic colitis, cigarette smoking is more prevalent compared to subjects without MC and first reports suggest that lung cancer is associated with MC[20-22]. The odds ratio (OR) for lymphocytic colitis and smoking (OR = 3.8) is higher than for collagenous colitis and smoking (OR = 2.4), although this difference was calculated on a small cohort of 120 patients with collagenous colitis, 70 patients with lymphocytic colitis, and 128 controls, and thus has to be verified in larger groups of patients[23]. Interestingly, it was additionally shown that MC occurs roughly 10 years earlier when the respective person is an active smoker, stressing the relevance of cigarette smoking to the pathophysiology of microscopic colitis. Beyond the strong results from association studies, it would be of great impact to learn whether cessation of smoking would cure MC or at least be beneficial to the patient's symptoms, and a prospective clinical trial answering this seems worthwhile. In addition to the inflammatory component in the pathophysiology of MC, there may be an additional neuronal component to pathophysiology. A recent study identified increased chromogranin A, chromogranin B and secretoneurin levels in feces of patients with collagenous colitis compared to relevant control groups. These observations may point to a neurogenic involvement in MC and, additionally, these stool markers are suggested to be helpful in discriminating MC from irritable bowel syndrome or classical inflammatory bowel disease[24].

The precise mechanism of diarrhea in these patients is not well understood. Factors that may play a role include bile salt induced injury, active chloride excretion, decrease in net sodium absorption, creation of a diffusion barrier by collagen band and increased local inflammatory mediators such as nitric oxide and prostaglandins. It remains unclear which of these results in the symptoms reported by patients. Two studies have looked at inflammatory cytokines in MC. Patients with MC seem to have a predominantly TH1 type cytokine profile with significant increases in interferon gamma, TNF-α and interleukin 15, as well as an increased inducible nitric oxide synthetase. Others have found increased levels of Transforming growth factor-β in patients with collagenous colitis[25].

CLINICAL PRESENTATION

Collagenous and lymphocytic colitis present with very similar symptoms and from a clinical perspective, there is no specific symptom or clinical feature that allows discriminating one from the other. Thus, the differentiation between the two entities is made by histology only. The typical clinical presentation involves chronic (either recurrent or intermittent) relapsing watery, nonbloody diarrhea. Symptoms may have been present for several months to 2-3 years before medical attention is sought and a diagnosis is made. Less frequent complaints include abdominal cramping, fecal incontinence and weight loss, although weight loss may be seen in 40% or more of patients with collagenous colitis. Incontinence is probably more a reflection of the advanced age of those individuals who are affected and patients with this problem may do well if treated with antidiarrheal agents[26,27]. The natural history of MC is variable. Many cases are self-limiting, with symptoms lasting a few weeks or months. Others may be symptomatic for years in a relapsing or continuous pattern. Although a small number of case reports have suggested that MC may lead to development of ulcerative colitis, a small case series of patients with MC showed that none developed ulcerative colitis or Cohn's disease after a follow-up of at least 6 years[28]. There are case reports on spontaneous and colonoscopy induced

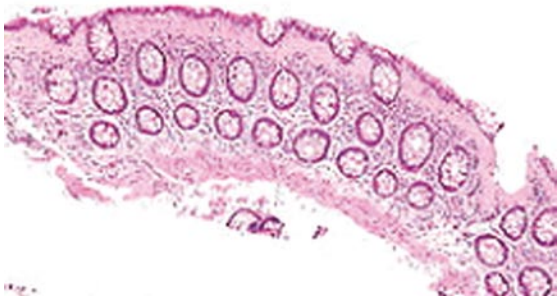

Figure 1 Micrograph of collagenous colitis, a type of microscopic colitis showing sub epithelial band of collagen H and E stain.

colonic perforations in patients with MC[29-30].

DIAGNOSIS/HISTOPATHOLOGY

The diagnosis of MC is dependent on (1) a convincing clinical history with other etiologies ruled out; (2) normal or near normal endoscopic and/or radiographic findings; and (3) endoscopic biopsies with histopathological findings consistent with MC.

The first step in the diagnostic process is a thorough history with particular attention paid to risk factors and the disease associated with MC. A complete history helps one to rule out other etiologies that may cause a similar clinical picture, such as IBD, celiac disease, diarrhea-predominant irritable bowel syndrome or infectious colitis.

Laboratory and radiographic investigations can be employed to rule out other entities on the differential diagnosis list but they are typically unremarkable.

Endoscopy with biopsy is necessary to arrive at the diagnosis. Colonoscopy generally reveals normal mucosal appearance. However, non-specific changes such as erythema, edema, abnormal vascular markings or even tears associated with perforation have been described. The hallmark of microscopic colitis is an increase in inflammatory cells (*i.e.*, lymphocytes) in colonic biopsies with an otherwise normal appearance and architecture of the colon. Inflammatory cells are increased both in the surface epithelium ("intraepithelial lymphocytes") and in the lamina propria. In lymphocytic colitis, these are the only abnormal features.

In collagenous colitis, the features of lymphocytic colitis are present, with the additional presence of a characteristic thickened sub epithelial collagen band which may be up to 30 μm thick (Figure 1)[31].

As the mucosa is not ulcerated or otherwise disrupted, the diarrhea generally does not contain blood or pus[32]. The diarrhea in collagenous colitis is likely due to inflammatory process and sub epithelial collagen serves as a cofactor in the role of a diffusion barrier and increased levels of immunoreactive prostaglandins E2 in stool water may lead to secretary diarrhea. Some cases may have fibrosis due to increased mucosal secretion of vascular endothelial growth factor[32,33]. One important question is how many biopsies need to be taken and how many biopsies are needed to confirm or rule out microscopic colitis. Numerous studies showed that the microscopic lesions can be skipped and therefore random multiple colonic biopsies should be taken[34].

Treatment

Treatment recommendations for MC are largely based on case reports and uncontrolled studies. Specific agents evaluated include 5-aminosalicylic acid (5-ASA), prednisone, immunomodulators, bismuth, probiotics and *Boswellia* extract. Small randomized controlled trials have shown that agents such as budesonide offer promise as an effective form of symptomatic therapy for both collagenous and lymphocytic colitis. As a first step in managing MC, an in depth medication history should be taken with potentially precipitating medications stopped where possible. Associated conditions such as celiac disease should be appropriately managed. In patients with mild symptoms, dietary restrictions like avoiding caffeine and lactose might be helpful.

Anti-diarrheal therapies

Non-specific anti-diarrheal therapies such as loperamide are commonly used in the management of MC. Retrospective studies have suggested benefit with doses ranging from 2 to 16 mg/d[34]. Due to the safety of this agent and the possibility of spontaneous remission, loperamide is the first-line therapy for MC.

Aminosalicylates

Uncontrolled retrospective series have suggested symptomatic improvement in up to 50% of patients with MC treated with mesalamine (5-ASA). A recent randomized trial of 64 MC patients compared mesalamine (800 mg *tid*) to mesalamine (800 mg *tid*) and cholestyramine (4 g/d). Treatment resulted in resolution of diarrhea in 84% overall after 2 wk. If treatment was continued over 6 mo, clinical and histological remission was achieved in 85% of those with lymphocytic and 91% of those with collagenous colitis. The number of relapsing patients after 6 mo of treatment was low and symptomatic relapses could be successfully retreated. Overall, the combination of mesalamine with cholestyramine was slightly superior[35,36].

Budesonide

Budesonide is currently the most promising treatment for collagenous colitis. Three trials involving 94 patients have shown that budesonide therapy (9 mg/d for 6-8 wk) compared to placebo resulted in statistically significant improvements in clinical symptoms and quality of life. A recent Cochrane database meta-analysis reported pooled OR of 12.3 for clinical response with budesonide with a number needed to treat of two. Although effective in the short-term, all trials showed a high rate (61%-80%) of relapse within 2 wk of budesonide cessation. Age < 60 years was a significant risk factor for relapse. Although there are no studies to support a tapering course of budesonide, many clinicians employ this in an effort to

minimize the likelihood of relapse.

One randomized controlled trial of budesonide for the treatment of lymphocytic colitis has been conducted. When compared to the placebo arm, the patients randomized to budesonide (9 mg/d × 6 wk) had a statistically significantly higher rate of remission (< 3 bowel movements per day) at 3 and 6 wk[37-41].

Prednisolone

A double-blind, placebo-controlled randomized trial of oral prednisolone 50 mg/d for 2 wk for collagenous colitis was inconclusive because of the low number of patients enrolled[42]. Studies examining the effect of prednisone in the treatment of lymphocytic colitis have not been performed.

Immunosuppressive therapy

Immunosuppressive therapy with azathioprine or methotrexate has been utilized in patients either refractory to corticosteroid therapy or corticosteroid dependent, but there are no randomized controlled trials to guide therapy with these medications.

Other therapies

Small clinical trials studying bismuth subsalicylate, *Boswellia serrata* extract, probiotics and empirical antibiotic treatment for collagenous and lymphocytic colitis look promising but cannot be suggested outside of such trials. Finally, case reports suggest that pentoxifylline, verapamil and subcutaneous octreotide might be treatment options, but their use cannot be recommended at this time. When medical therapy was unsuccessful and symptoms were very severe, surgical interventions, such as a temporary or permanent loop ileostomy or even a proctocolectomy, have been employed in smaller case series.

CONCLUSION

To conclude, the term microscopic colitis is now used to describe both lymphocytic and collagenous colitis and the condition should be kept in mind in any patient with unexplained watery nonbloody diarrhea with normal endoscopic findings. Biopsy is a must to rule out either form of microscopic colitis. Based on symptom severity and disease duration, a stepwise approach to treatment is suggested.

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3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

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5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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TOPIC HIGHLIGHT

WJGP 5th Anniversary Special Issues (2): Ulcerative colitis

"Mucosal healing" in ulcerative colitis: Between clinical evidence and market suggestion

Cristiano Pagnini, Francesca Menasci, Stefano Festa, Gianenrico Rizzatti, Gianfranco Delle Fave

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Abstract

In recent decades, the prominent role of endoscopy in the management of ulcerative colitis (UC) has been translated into the concept of mucosal healing (MH) as a fundamental therapeutic end-point. This is partially the consequence of growing evidence of a positive prognostic role of MH on the disease course and partially due to market cues indicating a higher rate of MH in patients treated by novel potent biologic agents. The aim of the present review is to clarify the current knowledge of MH in UC, analyzing the definition, the putative prognostic role and the association of MH with the current drugs used to treat UC patients. Because solid data about the management of UC patients based solely on the healing of the mucosa are not yet available, a tailored approach for individual patients that-considers the natural history of UC and the presence of prognostic indicators of aggressive disease is desirable. Consequently, unnecessary examinations and treatment would be avoided and restricted to UC patients who require the maximum amount of effort to affect the disease course in the short and long term.

Key words: Ulcerative colitis; Mucosal healing; Prognosis; Evidence

Core tip: In recent years, the concept that the management of ulcerative colitis patients should aim to heal the mucosa rather than resolve symptoms has been decisively proposed. Herein, we review the current evidence supporting this statement and analyze the possible practical implications in the current management of ulcerative colitis patients.

Pagnini C, Menasci F, Festa S, Rizzatti G, Delle Fave G. "Mucosal healing" in ulcerative colitis: Between clinical evidence and market suggestion. *World J Gastrointest Pathophysiol* 2014; 5(2): 54-62 Available from: URL: http://www.wjgnet.com/2150-5330/full/v5/i2/54.htm DOI: http://dx.doi.org/10.4291/wjgp.v5.i2.54

INTRODUCTION

A crucial topic that physicians have long faced in the management of ulcerative colitis (UC) patients is the identification of a reference parameter for the assessment of disease activity. Indeed, UC is a chronic inflammatory disease of the colon, characterized by limitation of the inflammation to the mucosa and the proximal extension of the disease starting from the rectum[1]. Indeed, the term "UC" comprises a heterogeneous condition with differing involvement of the colon in terms of its extension and the grade of inflammation, which in turn can lead to possible alterations of laboratory parameters and symptom occurrence and severity. The clinical, biochemical and mucosal alterations do not always directly correlate, and questions have been raised about which parameter should be used as the "gold standard" for disease activity assessment.

For the capacity to directly evaluate the colon, which is the target organ of the disease, endoscopy has been indicated as the more accurate tool to assess the activity of the disease, further supported by the possible misleading role of symptoms in the evaluation of UC patients[2,3]. Unfortunately, colonoscopy is an invasive, costly and time-consuming procedure, and the routine repetition of the examination is not feasible. Different objective surrogate parameters have been described to aid physicians in the correct evaluation of the activity state of patients with inflammatory bowel disease (IBD), including serological (*i.e.*, C-reactive protein) and fecal (*i.e.*, calprotectin, lactoferrin) markers[4], as well as clinical scores[5].

During the last decade, the addition to the IBD therapeutic arsenal of anti-TNF-α biologic drugs, which were formerly used in other chronic inflammatory conditions, has launched a "Copernican revolution" in the clinical approach to both Crohn's disease (CD) patients and UC patients. In fact, together with a rapid and consistent improvement of symptoms and laboratory parameters, such potent anti-inflammatory compounds have resulted in rapid and dramatic improvements of the intestinal mucosal lesions characteristic of IBD, as documented by endoscopic evaluation before and after the induction therapy[6]. Since this time, the relevance of the endoscopic activity of disease has been definitively stated, and "mucosal healing" (MH) has been proposed with increasing strength as a fundamental therapeutic goal of IBD treatment, claiming its prognostic relevance in the natural history of the disease[3]. Since the first studies that described the efficacy of Infliximab in CD patients[7,8], some 15 years have passed, and the therapeutic options for IBD patients have consistently expanded. At present, two biologic anti-TNF agents are currently approved in Europe for utilization in both CD and UC (Infliximab and Adalimumab), and some biologic agents have already shown efficacy in randomized clinical trials and are indicated for market release[9]. The emphasis on the efficacy of such novel drugs for the amelioration of mucosal inflammation has contributed to making the concept of MH a paramount therapeutic goal, and we are passing from a symptom-targeted to a mucosa-targeted approach in the management of IBD patients[10]. Several observations have contributed to encourage this shift in IBD management, outlining the relation between mucosal healing and the favorable long-term outcome of the disease in terms of reductions in flares, hospitalizations, the need for surgery and cancer incidence[11].

Although the MH concept has recently been particularly emphasized in CD, the importance of endoscopic remission in UC has been known for a long time[12]. In fact, the achievement of MH in UC appears of particular relevance for the localization of the disease (mucosal and limited to the colon), which renders the endoscopic examination relatively easier compared with CD, in which the inflammation is transmural and can potentially involve areas of the intestine not accessible to endoscopic inspection. Considering that more than half of UC patients present inflammation limited to the left side of the colon[13], the possibility that the involved areas can be easily scoped to evaluate MH in such patients is particularly tempting.

Nonetheless, specific treat-to-target studies addressing the effective role on the natural progression of the disease of a treatment strategy focused electively on the achievement of MH are still lacking. The relevance of MH to the management of UC, although intriguing and rational, remains to be firmly established. The possibility that the importance of MH would tend to be overrated due to the influence of sponsored trials underlining the association between MH and biologic drugs must be considered. Moreover, data coming from randomized clinical trials (RCTs) are usually not completely applicable to the "real-life" IBD population. In fact, a recent retrospective analysis of consecutive mild-moderate IBD patients at a United States tertiary referral center found that only 31.1% of patients would fulfill the inclusion criteria of the major RCTs of biologic agents and that the outcomes of patients fulfilling the criteria are significantly more favorable compared with those not meeting the criteria[14].

Besides scientific and commercial suggestions, a careful revision of the actual evidence in support of MH is essential. The risk of a blind and excessively enthusiastic adherence to the MH suggestion is concrete, and physicians need to be aware of the over-prescription of unnecessary endoscopic examinations and/or the over-treatment of patients. In an era of resource optimization, this would risk minimizing the same advantages that the MH strategy is claiming, *i.e.*, the reduction of disease costs by reducing complications and hospitalizations. Extensive systematic reviews of MH are already available in the literature (*i.e.*, Neurath *et al*[11]), and such a review is beyond the aim of the present work. Here, we intend to perform a synthetic and careful revision of the state-of-the art research on MH. To this end, we critically reviewed the definition of MH, the quality of the actual evidence of its prognostic relevance, and the capacity of the therapies currently used for UC to achieve MH, with the final goal of clarifying the potential correct application of the concepts of MH to the current practical management of patients affected by UC.

MH: DEFINITION

Although a standardized definition of MH has not been established, a practical currently accepted definition is "the complete resolution of the visible alterations or lesions, irrespective of their severity and/or type at baseline colonoscopy"[11]. Nonetheless, at present, an easy to use, validated and clinically relevant endoscopic score for UC activity evaluation is lacking, reflecting the complexity in measuring disease activity in UC[15]. In fact, although a great number of scoring systems have been developed (Baron score, Mayo score, Sutherland, Powell-Tuck and Rachmilewitz indices, among others)[16-24], none of them have been prospectively validated. The main problems

regarding the majority of the indices include the overlap of mucosal features (such as vascularity, granularity, erythema, friability, bleeding, and ulceration), leading to inter-observer variation in endoscopic evaluation, and the lack of clear and standardized thresholds for endoscopic remission or improvement. The Mayo Clinic endoscopy subscore has been the most commonly used in recent clinical trials, defining MH as a score of $\leqslant 1$ (normal mucosa or loss of vascular pattern, but no mucosal friability), when the endoscopy subscore was 2 or 3 at baseline. The problem of a standardized definition of MH is not theoretical but implies concrete and practical consequences. In fact, in recent clinical trials, heterogeneous definitions may have contributed to the higher rate of patients with MH when compared with that of patients achieving clinical remission[25], although alternative explanations are possible (*e.g.*, the simultaneous presence of irritable bowel syndrome, dysmotility). Moreover, a recent RCT testing the use of mesalamine in UC patients showed consistently different results after a revision of the endoscopic examination findings by a blinded central reader[26].

In further support of the aforementioned difficult evaluation of UC endoscopic activity, two novel scores have been very recently developed and prospectively validated, the Ulcerative Colitis Endoscopic Index of Severity and the Ulcerative Colitis Colonoscopic Index of Severity[27,28]. Data about the applicability of such new scores in clinical trials and in clinical practice are awaited and will hopefully aid the move toward a standardized definition of MH.

Recently, data indicating a prognostically relevant role for histologic activity in the mucosa of UC patients, in addition to the macroscopic activity, have opened the door to the concept of "histological MH", with the complete absence of clinical, laboratory, endoscopic and histological features of active inflammation[29]. Indeed, the term "mucosal healing" was initially proposed only for the disappearance of the inflammatory infiltrate in the histological examination[30]. At present, although some scoring systems for histologic activity have been described, none have been properly validated or commonly used, and therefore, the definition of histological MH remains without consensus.

MH: EVIDENCE FOR PROGNOSTIC RELEVANCE

The increasing relevance of the MH achievement in UC has been demonstrated by a growing body of data showing the different courses of the disease in patients with and without MH, with a reduction of complications such as flares as well as reductions in hospitalization, colectomy and cancer incidence in patients with MH.

As early as 1966, Wright *et al*[31] reported a higher relapse rate in patients who did not achieve MH after oral and rectal steroids when compared with patients who did achieve MH (40% *vs* 18%). In the ACT1 and ACT2 trials, patients treated with infliximab who exhibited MH at week 8 showed a higher rate of clinical remission at week 30 than patients without MH (48.3% *vs* 9.5%)[32]. Yamamoto *et al*[33] reported that UC patients who achieved clinical remission and MH after leukocytapheresis had a higher rate of sustained clinical response when compared with patients with only a clinical response (88% *vs* 41%). Ardizzone *et al*[34] showed that the lack of mucosal healing at 3 mo after the first corticosteroid treatment was the only factor associated with negative outcomes at 5 years (use of immunosuppressants, hospitalization and colectomy).

An observational study of the IBSEN cohort showed that in 513 UC patients, the colectomy rate was lower in patients with MH [defined by a simple endoscopic score of 0-1 (0, normal; 1, light erythema or granularity)] at a 5-year follow-up (2% *vs* 8%, $P < 0.05$)[35]. Similar results were shown by Soldberg *et al*[36], who reported a decrease in the colectomy rate in UC patients with MH at 1 year after diagnosis, regardless of the therapy used to achieve it, and in a post-hoc analysis of the ACT1/ACT2 trials conducted by Colombel *et al*[37], in which a Mayo Clinic endoscopy subscore of 0-1 in Infliximab-treated patients was related to a lower probability of colectomy than a score of 2-3 through a follow-up period of 54 wk. Interestingly, in the latter article by Colombel *et al*[37], MH in the placebo group did not show the same positive prognostic value as it did in the Infliximab-treated group, questioning the prognostic value of MH "*per se*" and suggesting that the drugs used to achieve the MH may play a specific role in the long-term outcome.

The increased risk of colorectal cancer incidence in UC patients is still a matter of debate[38]. Nonetheless, the inflammatory burden appears to be an important determinant, and consequently, MH is likely to reduce the risk. An Italian cohort study indicated a lower CRC risk at 17 years of follow-up in azathioprine (AZA)-treated UC patients with MH[39].

Recently, appealing data have indicated a possible prognostic role for histologic remission in terms of reductions in flares, surgery/hospitalization and CRC incidence, suggesting histologic remission as the ultimate therapeutic goal in UC management[29]. In fact, Bitton *et al*[40] have reported basal plasmacytosis at rectal biopsy as an independent predictor of early relapse in UC patients, and Bessissow *et al*[41] have described a higher rate of flares in patients with macroscopically healed mucosa but histologic activity when compared with patients with both the macro- and microscopic absence of disease. Nonetheless, correlations with macroscopic and microscopic activity are not always straightforward[42], and routine biopsies are not suggested by the current guidelines. At present, more evidence is needed before considering histological MH as a possible goal of treatment in UC patients.

MH: CURRENT THERAPIES

Biologic agents

As mentioned, the MH concept has been clearly defined

Table 1 Randomized clinical trial of biologic agent in ulcerative colitis and the relative mucosal healing rates

Ref.	Patients (*n*)	Treatment protocol duration	Evaluation time from baseline	MH rate
Rutgeerts *et al*[32]	728	IFX 5 or 10 mg/kg every 8 wk	Week 8	60.7% IFX
		Placebo		32.3% placebo
		30 wk (ACT2)	Week 30	50.6% IFX
				27.4% placebo
		54 wk (ACT1)	Week 54	46.0% IFX
				18.2% placebo
Panaccione *et al*[50]	231	AZA 2.5 mg/kg	Week 16	37% AZA
		IFX 5 mg/kg		55% IFX
		IFX 5 mg/kg + AZA 2.5 mg/kg		63% AZA + IFX
		16 wk		
Sandborn *et al*[25]	494	ADA 160/80 and then 40 mg eow	Week 8	41.1% ADA
		Placebo		31.7% placebo
		52 wk	Week 52	25.0% ADA
				15.4% placebo
Reinisch *et al*[43]	390	ADA 160/80 mg or 80/40 mg at weeks 0 and 2 followed by 40 mg at weeks 4 and 6	Week 8	46.9% ADA (160/80)
		Placebo		37.7% ADA (80/40)
				41.5% placebo
		52 wk	Week 52	54% ADA
Feagan *et al*[44]	225	VED 300 mg at week 0 and 2 and then every 4 or 8 wk	Week 6	40.7% VED
		Placebo		24.8% placebo
		52 wk	Week 52	56% VED (every 4 wk)
				51.6% VED (every 8 wk)
				19.8% placebo
Sandborn *et al*[45,46]	774	GOL 400/200 or 200/100 mg at weeks 0 and 2 followed by 50 mg or 100 mg every 4 wk	Week 6	45.1% GOL (400/200)
		Placebo		42.3% GOL (200/100)
				28.7% placebo
		54 wk	Week 54	42.4% GOL100 every 4 wk
				41.7% GOL 50 every 4 wk
				26.6% placebo

MH: Mucosal healing; IFX: Infliximab; ADA: Adalimumab; AZA: Azathioprine; VED: Vedolizumab; GOL: Golimumab.

only in the biologic era, and the trials of biologic drugs present a better evaluation of this aspect than previous studies. In particular, the MH definition has been standardized by the utilization of the Mayo endoscopic subscore, which identifies as MH as a score of 0 or 1. However, MH has always been considered as a secondary end-point in clinical trials, and studies still present heterogeneity in terms of inclusion criteria (and, therefore, baseline endoscopic severity), design and follow-up. Nonetheless, the MH rates in the short (induction) and long term (maintenance) are consistent and significantly superior to those of placebo in all studies (Table 1), which is even more remarkable considering the baseline severity of the UC patients included, although, in most of the studies, MH was only observed in a minority of the patients[25,32,43-46]. Moreover, as mentioned, patients in RCTs are superselected, and the results may be not directly applicable to the "real-life" IBD population.

Azathioprine

From the first early report by Jewell *et al*[47] of increased MH after 4 wk in UC patients treated with corticosteroids plus AZA *vs* corticosteroids plus placebo (92% *vs* 71%, *P* = ns), few studies with a limited number of patients have addressed MH rates in AZA-treated UC patients. In all of the reported studies, MH was a secondary end-point, and the MH definition, base-line endoscopic activity, timing of the endoscopic evaluation and concomitant therapies differed; therefore, conclusive results are hard to extrapolate.

With the aforementioned limitations, Paoluzi *et al*[48] reported 57% and 45% rates of MH in UC patients treated with AZA at 6 mo (*n* = 42 patients) and 4 years (*n* = 22 patients), respectively, and a similar 6-mo rate was reported by Ardizzone *at al*[49] [19/36 patients treated with AZA (53%) *vs* 7/36 of patients treated by 5ASA (19%)]. Recently, a study by Panaccione *et al*[50] (available only in abstract form) reported a 36% MH rate in patients treated with AZA in monotherapy and a 63% MH rate in patients treated with AZA plus Infliximab at 4 mo, with nearly 80 patients per group, indicating that combination therapy may increase the rate of MH.

Corticosteroids

Unlike CD, in which corticosteroids are traditionally considered ineffective for the achievement of MH[51], corticosteroids may induce MH and a clinical response in UC. The first evidence supporting a favorable role of corticosteroids in inducing MH dates back to 1954, when Truelove reported a double-blind placebo-controlled randomized multicenter trial of 120 UC patients and demonstrated higher rates of MH in the oral cortisone (100 mg/d) group

than in the placebo group (30% *vs* 10%) within 6 wk[16].

In the last six decades, a great number of studies have reported positive effects of corticosteroid therapy on the improvement/resolution of mucosal alterations in UC, irrespective of the route of administration (oral or rectal) and the type of corticosteroids (traditional systemic steroids or agents with low systemic availability)[52-59]. Generally, a certain discrepancy between the clinical and endoscopic responses was present in the majority of the studies evaluating MH in UC after corticosteroid treatment. A meta-analysis by Marshall *et al*[55] examining the role of rectal corticosteroid preparations showed similar clinical (approximately 45% of cases) and endoscopic (approximately 33% of cases) remission rates for conventional corticosteroids (hydrocortisone, prednisolone, methylprednisolone and betamethasone) and topically active corticosteroids (beclomethasone, budesonide and prednisolone metasulphobenzoate). Recently, Ardizzone *et al*[34], in a study of 157 consecutive newly diagnosed UC patients, explored the potential prognostic significance of a 3-mo clinical and endoscopic response after the first course of corticosteroid treatment. After 3 months, 60 patients (38.2%) had a complete clinical and endoscopic response, 39 (24.8%) had a clinical but not an endoscopic response, and 58 (36.9%) had no response. Interestingly, failure to achieve endoscopic remission at the end of the first course of steroids was related to a more aggressive disease behavior.

Data obtained from the use of topical steroids present a reduced variability between clinical and endoscopic responses. Indeed, in a recent meta-analysis exploring the efficacy of rectal beclomethasone dipropionate, the clinical and endoscopic rates of improvement or remission were similar (65.3%) and concordant, although in the four trials considered for the meta-analysis, a clear definition and evaluation of mucosal healing were lacking[60].

Several problems arise in the attempt to analyze and compare the results of the above-mentioned studies. Diversity in the timing of endoscopy and in the use of endoscopic indices (*e.g.*, Sigmoidoscopic score, Rachmilewitz index, Baron score) along with the lack of a univocal MH definition, possible inter-observer variations or heterogeneity of the included patient cohorts may have generally contributed to consistent variability in the MH rates in steroid trials.

Aminosalicylates

Mesalamine was approved by the Food and Drug Administration in late 1987, and since this time, it has become the cornerstone therapy for mild-moderate UC[61]. Mesalamine can be administered orally and/or topically, and it is present on the market in different formulations specific to both methods of administration. Many studies show the ability of mesalamine to induce MH. A recent meta-analysis of 49 studies has concluded that MH is achieved in approximately 37% and 50% of patients treated with oral and topical mesalamine, respectively[62]. Nonetheless, the results from single studies are dramatically different, ranging from approximately 0% to 77% for oral mesalamine[23,63] and from approximately 10% to 93% for topical formulations[54,64]. This variability may be attributed to the different definitions of MH, but this is unlikely to be the only reason. While MH rates do not appear to be related to the release mechanisms of oral mesalamine[62], in accordance with previous studies reporting similar effectiveness between different formulations[61,65,66], studies continue to present great heterogeneity in terms of total dose in grams, disease extension, months of follow up and endoscopic score at baseline. Notably, the MH rates in placebo groups are reported to be high, up to 46% in a study of oral placebo *vs* oral mesalamine at 8 wk[63] and 26%-37% in a study of topical placebo *vs* topical mesalamine after 6 wk[67]. Moreover, in studies with therapeutic regimens of adequate dose and duration, the MH rate appears to be higher[68-70], and the lack of achievement of MH in patients with clinical remission has been indicated as a possible negative prognostic factor for relapse occurrence[71].

CONCLUSION

After the emergence of novel biologic therapies for UC, the old concept of the relevance of the endoscopic activity of disease has been translated into the new concept of MH as the therapeutic goal to achieve. Although this idea has been supported by a growing body of scientific evidence indicating the favorable prognostic value of a healed mucosa in the natural history of UC, it is also suggested commercially, as a high rate of MH is claimed when utilizing the new biologic agents. Indeed, endoscopic evaluation appears to be the "gold standard" for the evaluation of disease activity in UC patients, and healing of the mucosa is likely to be an important factor for the control of the disease in the short and long term. However, specific studies showing the superiority of a management based solely on MH over the "traditional" approach are lacking. To date, most of the evidence supporting the prognostic relevance of MH comes from studies in which MH is not considered as the primary endpoint as well as from retrospective investigations. In the present study, we provocatively addressed the issue of the relevance of MH for UC patients management. A careful review of the current evidence regarding MH in UC shows that, due to the high heterogeneity of the available studies (particularly for those from the pre-biologic era), crucial points are still far from being conclusively determined, including the MH definition, the expected rate of MH with the current medication, and whether a systematic assessment of MH and an optimization of therapy based on MH alone would improve long-term disease outcome. Moreover, the prognostic value of MH "*per se*" needs to be investigated to clarify whether the current drugs may be safely reduced or interrupted after MH achievement. The latter issue may also present consistent economic implications regarding the elevated cost of long-term maintenance therapy with biologic drugs. However, in most cases, MH appears to be achiev-

able only in a minority of UC patients and most likely with the utilization of potent and potentially dangerous therapeutic regimens. In the near future, the development of novel drugs and an increase in our knowledge of the complexity of IBD are desirable, as they may increase the efficacy of our therapeutic approach to the disease.

Notably, going back to the natural history of the disease, more than one-half of UC patients have a benign disease course, while up to one-third are likely to experience frequent flares and potentially dangerous complications. In fact, the large population study by Solberg *et al*[36] (IBSEN cohort), which evaluated the first 10 years of the disease course in a population of 519 patients with UC, highlighted an overall good prognosis. Their study showed that at 10 years, more than half of patients were in remission or had mild disease, while 37% and 6%, respectively, reported chronic intermittent and chronic continuous symptoms. In a large Danish cohort study, approximately one-third of patients had no flares within 10 years after the first attack of UC. Moreover, the cumulative probability of having a course without relapses after 10 years in patients in remission is 40%-60%[72]. However, the colectomy rate is estimated to vary from 8.7% to 30% in different populations[72-74], and after the first relapse, the cumulative rates of a second course of systemic steroids are 13%, 41% and 48% at 1, 5 and 10 years, respectively[36].

In times of resource optimization, the ideal disease management would imply an aggressive treatment and endoscopic follow-up for the achievement of MH in patients with an unfavorable disease course. Accordingly, together with a better definition of the MH concept and its specific role in the management of UC patients, further research for the characterization of clinical and/or genetic features predictive of an aggressive behavior of the disease is urgently needed. Similarly, the identification and the implementation of clinical and laboratory parameters strongly correlated with the endoscopic activity, such as clinical scores, to better follow-up these patients, appear to be of relevance[75]. Consequently, it is advisable that the aforementioned shift from a symptoms-based to a mucosa-based approach in the management of UC patients would not result in a trend to over-scope and/or over-treat patients for the achievement of MH. Indeed, because more solid evidence will be available regarding the role of MH, a rational approach to UC patients should reserve close monitoring and more potent therapies for "high-risk" patients, overcoming the dualism between symptom- and mucosa-targeted approaches and focusing increasingly on a "patient-based" approach.

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TOPIC HIGHLIGHT

WJGP 5th Anniversary Special Issues (2): Ulcerative colitis

Implication of miRNAs for inflammatory bowel disease treatment: Systematic review

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Abstract

Inflammatory bowel disease (IBD) is believed to develop *via* a complex interaction between genetic, environmental factors and the mucosal immune system. Crohn's disease and ulcerative colitis are two major clinical forms of IBD. MicroRNAs (miRNAs) are a class of small, endogenous, noncoding RNA molecules, and evolutionary conserved in animals and plants. It controls protein production at the post-transcriptional level by targeting mRNAs for translational repression or degradation. MiRNAs are important in many biological processes, such as signal transduction, cellular proliferation, differentiation and apoptosis. Considerable attention has been paid on the key role of miRNAs in autoimmune and inflammatory disease, especially IBD. Recent studies have identified altered miRNA profiles in ulcerative colitis, Crohn's disease and inflammatory bowel disease-associated colorectal cancer. In addition, emerging data have implicated that special miRNAs which suppress functional targets play a critical role in regulating key pathogenic mechanism in IBD. MiRNAs were found involving in regulation of nuclear transcription factor kappa B pathway (*e.g.*, miR-146a, miR-146b, miR-122, miR-132, miR-126), intestinal epithelial barrier function (*e.g.*, miR-21, miR-150, miR-200b) and the autophagic activity (*e.g.*, miR-30c, miR-130a, miR-106b, miR-93, miR-196). This review aims at discussing recent advances in our understanding of miRNAs in IBD pathogenesis, their role as disease biomarkers, and perspective for future investigation and clinical application.

Key words: Crohn's disease; Inflammatory bowel disease; MicroRNA; Treatment; Ulcerative colitis; Biomarker

Core tip: MicroRNAs (miRNAs) are a class of small, noncoding RNA molecules that post-transcriptionally regulate gene and protein expression. Recent studies have identified altered miRNA profiles in inflammatory bowel disease (IBD). Special miRNAs which suppress functional targets have been found to play a critical role in regulating key pathogenic mechanism in IBD. In this review, we discuss the possibility to use miRNAs as biomarkers and therapeutic target in IBD.

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INTRODUCTION

Inflammatory bowel disease (IBD) refers to chronic remittent or progressive inflammatory conditions that may affect the entire gastrointestinal tract. Crohn's disease (CD) and ulcerative colitis (UC) are two major clinical forms of IBD[1]. The incidence and prevalence of IBD is continuously increasing over the past decades in different regions around the world[2]. Although the precise pathogenesis of IBD remains obscure, several reports have

indicated that dysfunction of the mucosal immune system which develops *via* a complex interaction between genetic factors, the host immune system and environmental factors plays an important role in its etiology[3]. The chronic inflammation of IBD is associated with marked molecular changes in gene and protein expression[4]. So small molecules targeted at the pathways involving in these processes may be potential for IBD diagnosis and treatment.

MicroRNAs (miRNAs) are considered as promising candidate. They are a class of single-stranded non-coding RNA molecules on an average 22 nucleotides long[5], and are highly conserved throughout evolution[6] and discovered in all eukaryotic cells except fungi[7]. MiRNAs regulate gene expression both at a transcriptional and translational level[8], and mediate post-transcriptional gene silencing by directly binding to the 3' untranslated region (UTR) of target mRNA. Depending on the level of sequence complementarity between miRNA and target site, mRNA transcripts targeted by miRNAs are either silenced if the base-pair match is imperfect or degraded if there is an identical base-pair match[9]. The mRNAs inhibited by miRNAs move to cytoplasm and accumulate in cytosolic processing bodies until they are eventually degraded[10]. Each miRNA can target hundreds of genes, and a particular gene is usually the target of multiple miRNAs, adding complexity to the regulation of gene transcriptional network[11]. It has been reported that miRNAs play an important role in many biological processes, such as signal transduction, cellular proliferation, differentiation, apoptosis and immune response[12,13]. Recently, miRNAs have been recognized as critical elements in the regulation of the innate and adaptive immune responses, and changes in miRNAs expression are related to many autoimmune diseases, including systemic lupus erythematosus, rheumatoid arthritis, psoriasis and IBD[14-17].

In this review, we summarize the current understanding of the connection between miRNAs and IBD. We mainly focus on special dysregulated miRNAs in CD and UC, which lead to inappropriate expression of targeted mRNA and may contribute to IBD pathogenesis, diagnosis and treatment. Table 1 summarizes the altered miRNAs involved in IBD and their mRNA targets.

MIRNAS REGULATE NUCLEAR TRANSCRIPTION FACTOR KAPPAB PATHWAY

The nuclear transcription factor kappaB (NF-κB) was identified as one of the important regulators in the immune system and inflammatory diseases[18]. NF-κB is markedly induced in IBD patients and strongly influences the course of mucosal inflammation through its ability to promote the expression of various pro-inflammatory genes[19]. Nucleotide-binding oligomerization domain 2 (NOD2) was found to be the first IBD susceptibility gene[20], which is mainly expressed in Paneth cells, monocytes, macrophages, dendritic cells and some types of intestinal epithelia cell[21]. NOD2 can be activated by muramyl dipeptide (MDP), a component of bacterial cell wall, which induces the activation of NF-κB[22].

MiR-146a was reported to regulate gut inflammation *via* NOD2-sonic hedgehog (SHH) signaling[23]. SHH signaling is an important pathway that maintains gut homeostasis and directs gut development. The expressions of NOD2-induced iNOS and NO were increased in MDP-treated macrophages, which further induced the level of miR-146. Promoter luciferase analysis with miR-146a promoters revealed that NF-κB was a critical transcription factor that regulate NOD2 mediated expression of miR-146a. NOD-2 induced miR-146a target NUMB, a negative regulator of SHH signaling, alleviating the suppression of SHH signaling and subsequently increasing the pro-inflammatory cytokines expression.

Feng *et al*[24] proved that up-regulation of miR-126 may contribute to pathogenesis of UC by targeting IκBα. They found miR-126 was significantly increased in active UC tissues compared to healthy controls. IκBα, an inhibitor of NF-κB pathway and the target of miR-126, was markedly decreased in active UC tissues. The expression of miR-126 and IκBα were inversely correlated in patients with active UC. MiR-126 could inhibit the level of IκBα in HT29 cells. They further demonstrated that miR-126 may activate NF-κB signaling pathway by targeting IκBα and contribute to the development of UC. Another study showed that the anti-inflammatory activities of the red wine polyphenolics were, at least in part, mediated by the induction of miR-126[25]. CAMs, such as intracellular cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), are expressed on the surface of fibroblasts[26]. It has been demonstrated that the expression of ICAM-1 was increased in CD patients[27] and inhibition of CAMs could suppress various forms of experimental inflammatory and immune responses in colon fibroblast cells[28]. VCAM-1 has been confirmed as one of the targets of miR-126 before[29]. Angel-Morales *et al*[25] found the polyphenolic red wine extract (WE) exerted an anti-inflammatory effect in LPS-stimulated human colon-derived CCD-18Co myofibroblast cells through inactivating NF-κB and down-regulating a wide range of downstream pro-inflammatory genes including tumor necrosis factor (TNF)-α, interleukin-6 (IL-6) and CAMs. Furthermore, they found the up-regulation of miR-126 was induced by WE in CCD-18Co cells and protected human colon cells from inflammation through targeting VCAM-1.

MiR-122 was found dysregulated in association with CD progression[30]. Chen *et al*[31] identified NOD2 as a target of miR-122. Overexpression of miR-122 in LPS-stimulated HT-29 cells inhibited LPS-induced apoptosis and down-regulated LPS-induced NOD2 expression. Pretreatment with miR-122 in LPS-stimulated HT-29 cells decreased the pro-inflammatory cytokines and increased the anti-inflammatory cytokines by targeting NOD2-induced NF-κB signaling pathway. Taken together, miR-122 might decrease intestinal epithelial cell injury in Crohn's disease by targeting NOD2. Besides regulating the activation of NF-κB pathway, Ye *et al*[32] demonstrated

Table 1 List a core set of altered microRNAs involved in inflammatory bowel disease and their mRNA targets

miRNA	Target mRNA	Net effect	Ref.
Increased expression			
miR-146a	NUMB	SHH signaling upregulation	[23]
miR-146b	Siah2	NFκB signaling upregulation	[40]
miR-126	IκBα	NFκB signaling upregulation	[24]
	Vascular cell adhesion molecule-1	Suppresses proinflammatory cytokines	[25]
miR-122	Nucleotide-binding oligomerization domain 2	Decreases intestinal epithelial cell injury	[31]
	Occluding	Intestinal permeability upregulation	[32]
miR-132	AChE	Decreases circulation AChE activity	[37]
miR-21	RhoB	Impairment of tight junctions	[52,53]
miR-150	c-Myb	Promotes apoptosis	[54]
miR-141	CXCL12β	Regulates leukocyte migration	[62]
miR-106b	ATG16L1	deregulation of autophagy	[67,68]
miR-196	IRGM	deregulation of autophagy	[70]
miR-30c	ATG5	inhibition of autophagic activity	[69]
miR-130a	ATG16L1	inhibition of autophagic activity	[69]
Decreased expression			
miR-10a	IL-12/IL-23p40	Regulates intestinal homeostasis	[43]
miR-124	STAT3	Promotes inflammation	[48]
miR-200b	ZEB1, SMAD2	Regulates epithelial-mesenchymal transition	[59,60]
miR-192,miR-495, miR-512,miR-671	NOD2	NFκB signaling upregulation	[34]

NF-κB: Nuclear transcription factor kappa B; NOD2: Nucleotide-binding oligomerization domain 2; AChE: Acetylcholinesterase; IRGM: Immunity-related GTPase family; IL: Interleukin; ZEB1: Zinc finger E-box binding homeobox 1; SMAD2: SMAD family member 2.

the involvement of miR-122 in the regulation of intestinal epithelial tight junction (TJ) permeability. Deficient intestinal epithelial TJ barrier, characterized by the increase of intestinal permeability, has been demonstrated to contribute to the development of IBD as an important pathogenic factor[33]. MiR-122 was significantly increased in TNF-α-stimulated Caco-2 cells and induced the increase in Caco-2 TJ permeability by targeting occluding. The up-regulation of intestinal permeability by miR-122 was proved *in vivo* as well[32]. Based on the two studies, miR-122 plays a complex and controversial role in the development of IBD.

Chuang *et al*[34] showed that NOD2 expression is regulated by miRNAs in HCT116 cells. They found that MDP could induce the expression of NOD2 and activate the NF-κB signaling pathway in HCT116 cells. MiRNAs targeted NOD2, such as miR-192, miR-495, miR-512 and miR-671, were significantly decreased in MDP-stimulated HCT116 cells, which had an inversely correlation with the expression of NOD2. Overexpression of these NOD2-associated miRNAs in MDP-stimulated HCT116 cells inhibited the activity of NF-κB and the downstream pro-inflammatory cytokines, IL-8 and CXCL3.

MiR-132 was a potential regulator of acetylcholinesterase (AChE) activity in inflammatory condition and was shown to target AChE to reduce its activity *in vitro* and in mouse models[35]. Acetylcholine (ACh) activates its receptor on macrophage through which it interrupts the nuclear translocation of NFκB and suppresses the production of pro-inflammatory cytokines[36]. Maharshak *et al*[37] found miR-132 had an anti-inflammatory effect on the development of IBD. MiR-132 level was significantly upregulated in biopsies from patients with IBD compared with controls. In accordance with this, circulation AChE activity was significantly lower in patients with IBD suffering from moderate-severe disease. These data implicated a possible regulation of AChE activity by increased miR-132 levels, which eventually ameliorated inflammation in patients with IBD.

Although NFκB was originally thought to be an almost exclusively pro-inflammatory player in the setting of IBD, its role in epithelial cells was confirmed more controversial. Several studies using knockout mice with defective NF-κB activation have demonstrated an anti-inflammatory function of NFκB in colonic epithelial cells[38,39]. Nata *et al*[40] showed that miR-146b, another member of miR-146 family, can alleviate intestinal injury in mouse colitis *via* the activation of NF-κB and the improvement of epithelial barrier function. MiR-146b was found significantly up-regulated in IL-10 deficient mice. The whole sequence of miR-146 was intraperitoneally administered to the dextran sodium sulfate (DSS)-induced colitis mouse. Overexpression of miR-146b in DSS-induced colitis mouse activated NFκB, relieved intestinal inflammation, improved epithelial barrier function, and increased the survival rate. Furthermore, the protective effect of miR-146b on mouse with DSS-induced colitis was negated by inhibition of the NFκB pathway. Siah2, which was the target of miR-146b, promoted ubiquitination of TRAF proteins upstream of NFκB. It suggested that miR-146b up-regulated NFκB *via* suppressing siah2, which finally improved intestinal inflammation.

MIRNAS REGULATE IL-23/IL-23R PATHWAY

IL-23, a heterodimeric cytokine comprising IL-12p40

and IL-23p19, is produced by activated macrophages, monocytes, DCs and endothelial cells. IL-23 receptor is composed of IL-12Rβ1 (shared with the IL-12 receptor) and the specific IL-23R subunit. IL-23 acts on the IL-23 receptor and promotes expansion and maintenance of Th17 cells, which secrete the pro-inflammatory cytokine IL-17 and have been implicated in the pathogenesis of many chronic inflammatory disorders, including IBD[41,42]. MiRNA was considered as a new mechanism in regulating the IL-23/TH17 pathway and subsequent downstream IL-17 production in IBD.

Xue *et al*[43] found much lower expression of miR-10a in intestinal epithelial cells and dendritic cells of specific pathogen-free mice compared to germ-free mice. IL-12/IL-23p40 was identified as a target of miR-10a. They further demonstrated that microbiota negatively regulated host miR-10a expression by targeting IL-12/IL-23p40, which may contribute to the maintenance of intestinal homeostasis.

IL-23R gene variants have been identified as risk factors for IBD[44]. The rs10889677 variant in the 3'UTR region of *IL-23R* gene which led to a loss of binging capacity for let-7e and let-7f displayed increased expression of IL-23R[45]. It means this mutation sustained IL-23 signaling and contributed to chronicity of IBD. Furthermore, Li *et al*[46] showed let-7f down-regulated the expression of IL-23R and its downstream cytokine IL-17 by targeting IL-23R.

MIRNAS REGULATE IL-6/STAT3 PATHWAY

Previous studies have shown the importance of the IL-6/STAT3 signaling pathway in IBD. Inhibition of IL-6/STAT3 cascades results in the suppression of acquired immune mediated colitis[47]. Koukos *et al*[48] found miR-124 were significantly decreased in colon tissues from children with UC and mice with experimental colitis, and the levels of STAT3 and its regulated genes were up-regulated simultaneously. They demonstrated reduced levels of miR-124 in colon tissues of pediatric patients with active UC might increase expression and activity of STAT3 by direct binding to its 3'UTR, which could promote inflammation and the pathogenesis of UC in children.

MIRNAS REGULATE INTESTINAL EPITHELIAL BARRIER FUNCTION

The intestinal mucosal barrier, of which the intestinal epithelial cells are the most integral part, maintains a delicate balance between absorbing essential nutrients while preventing the entry and responding to harmful subjects[49]. Dysfunction of intestinal epithelial barrier has been extensively reported in IBD[49,50].

Disruptions of important elements of the intestinal barrier in IBD lead to permeability defects[51]. There were two studies showed that miR-21 played a pro-inflammatory role in IBD by impairing intestinal barrier function. Yang *et al*[52] found levels of miR-21 were up-regulated in both the mucosal and serum of patients with UC. RhoB, which was the target of miR-21 and involved in modulating intestinal epithelial permeability, was found significantly decreased in the patients with UC. They demonstrated that overexpression of miR-21 in patients with UC and Caco-2 cells impaired intestinal tight junction integrity and morphology through targeting RhoB. Similarly, Shi *et al*[53] reported that miR-21 was overexpressed in IBD patients, IL-10 KO mice and DSS-treated mice. MiR-21 knockout (KO) mice was less susceptible to experimental colitis and had more ameliorative inflammatory responses than wild type (WT) mice. Moreover, the increase of Intestinal permeability and epithelial cells apoptosis induced by DSS were attenuated in miR-21 KO mice.

Bian *et al*[54] found miR-150 was significantly elevated, whereas c-Myb, a target of miR-150, was strongly decreased in colon tissue of UC patients and DSS-treated mice. Overexpression of miR-150 in HT29 cells enhanced cell apoptosis through targeting c-Myb, which damaged intestinal epithelial barrier.

Epithelial-to-mesenchymal-transition (EMT) is characterized by losing epithelial cell markers such as E-cadherin and gaining mesenchymal proteins including vimentin, which enhances invasiveness, migratory capacity and production of cell-extracellular matrix components[55,56]. Recent studies demonstrated that EMT contributed to the loss of intestinal epithelial cells (IECs) and subsequent increased intestinal paracellular permeability and decreased intestinal epithelial barrier function[57,58]. Chen *et al*[59] found miR-200b significantly decreased in inflamed mucosa in IBD patients, which was positively correlated to the expression of E-cadherin and negatively correlated to the level of TGF-β1 and vimentin. Overexpression of miR-200b in TGF-β1-stimulated IEC-6 cells increased E-cadherin and decreased vimentin through targeting zinc finger E-box binding homeobox 1 and SMAD2 respectively, which prevented TGF-β1-induced EMT. Intestinal fibrosis is a common serious complication of CD. In another study, they demonstrated that miR-200b could partially protect intestinal epithelial cells from fibrogenesis by suppressing EMT *in vitro*[60]. In summary, miR-200b played a potential role in maintaining intact of intestinal epithelium through inhibiting EMT and improving pathophysiology and clinical outcomes of IBD.

MIRNAS REGULATE COLONIC EPITHELIAL CELL-DERIVED CHEMOKINE EXPRESSION

The expression of intestinal epithelial-derived CXC and CC chemokines is increased in IBD[61]. Huang *et al*[62] found up-regulated level of miR-141 was inversely correlated with CXCL12β in the epithelial cells of the inflamed colon tissues from CD patients and mice with experimental colitis.They further demonstrated that miR-141 directly regulated CXCL12β expression and leukocyte migration

mediated by CXCL12β. Additionally, overexpression or knockdown of miR-141 in the colon of mice with experimental colitis regulated leukocyte infiltration and alleviated or aggravated intestinal inflammation, respectively. Wu *et al*[63] found miR-192 was decreased in active UC and demonstrated an inverse relationship between miR-192 and MIP-2 (CXCL2).

MIRNAS REGULATE AUTOPHAGY

Autophagy, which is involved in recycling cellular organelles for the survival of cell, is one mechanism for maintaining cellular hemostasis. Autophagy in the intestinal epithelium is considered to behave as a defensive strategy for clearance of intracellular microorganisms, and the impairment of autophagy results in intestinal epithelial dysfunction and contributes to IBD pathogenesis[64]. *ATG16L1* and *IRGM*, two genes associated with autophagy, have been identified as CD susceptibility genes by genome-wide association studies[65,66]. Some studies showed that miRNA-mediated change in the expression of autophagy gene may result in autophagy dysfunction and involve in the pathogenesis of IBD.

Lu *et al*[67] found that silencing of Dicer1 enhanced autophagy-related gene (ATG) protein levels and autophagosome formation in cells, indicating that miRNAs may be implicated in the regulation of autophagy. MiR-106b and miR-93, which target ATG16L1, both reduced levels of autophagy in epithelial cells. MiR-106b could also inhibit autophagy-dependent clearance of CD-associated adherent-invasive Escherichia col (AIEC) in epithelial cells. Inflamed mucosae from subjects with active CD exhibited more overexpressed miR-106b and lower expression of ATG16L1 when compared with controls. These results suggested that CD patients with miR-106b and miR-93 mediated down-regulation of ATG16L1 expression might manifest an altered antibacterial activity of CD-associated intracellular bacteria in epithelial cells and subsequently affected the outcome of intestinal inflammation. Similarly, Zhai *et al*[68] showed miR-106b targeted ATG16L1 and modulated autophagic activity in HCT116 cells. Their results further indicated that miR-106a and miR-106b could influence the expression of other autophagy-related genes and had a widespread modulating effect on the autophagy pathway.

Nguyen *et al*[69] proved miR-30c and miR-130a directly regulated the expression of ATG5 and ATG16L1, respectively, by targeting their 3'UTRs. They found miR-30c and miR-130a expression were increased and ATG5 and ATG16L1 mRNA expression were decreased in non-inflamed or inflamed ileal CD biopsy specimens compared with normal controls. Similarly, the expression of miR-30c and miR-130a were inversely correlated with ATG5 and ATG16L1 in intestinal epithelial T84 cells infected with the AIEC. NF-κB pathway was activated in AIEC infected T84 cells, which induced the up-regulation of miR-30c and miR-130a and consequently inhibited the expression of ATG5 and ATG16L1. The inhibition of autophagic activity by miR-30c and miR-130a increased AIEC persistence within T84 cells and enhanced pro-inflammatory cytokines production. Furthermore, they demonstrated inhibition of miR-30c and miR-130a *in vivo* suppressed AIEC-induced down-regulation of ATG5 and ATG16L1 expression and increased autophagic activity, leading to more efficient intracellular bacteria clearance and decreased inflammation.

Brest *et al*[70] demonstrated that the association of IRGM with CD arised from a miRNA-based alteration in IRGM regulation which led to the deregulation of autophagic efficacy. They found a synonymous variant in IRGM (c.313C > T), which was classified as non-causative before, altered a binging site for miR-196. MiR-196, was overexpressed in the inflammatory intestinal epithelia of patients with CD and down-regulated the IRGM protective variant (c.313C) but not the risk-associated allele (c.313T). Subsequent deregulation of IRGM-dependent autophagy compromised control of intracellular replication of CD-associated AIEC and affected the outcome of intestinal inflammation.

MIRNAS ASSOCIATION WITH IBD CARCINOGENESIS

The development of IBD-associated dysplasia and colorectal cancer represents a major complication in patients with IBD[71,72]. The important role miRNAs played in carcinogenesis is becoming clearer because miRNAs have been referred to the regulation of cancer-related cellular processes, including differentiation, apoptosis, cell cycle progression and immune function[10]. Growing evidence implicated that miRNAs are also involved in IBD-associated carcinogenesis.

Ludwig *et al*[73] showed up-regulated level of miR-21 in IBD-associated dysplastic lesions compared to active IBD patients, which was inversely correlated with the expression of PDCD4, a newly characterized tumor suppressor gene. Olaru *et al*[74,75] found expressions of miR-224 and miR-31 increased successively at each stage of IBD progression from non-inflamed to inflamed non-neoplastic, dysplastic and finally cancerous mucosae. MiR-224 and miR-31 levels could accurately discriminate normal or chronically inflamed IBD tissues from cancers. They further identified miR-224 regulated cell cycle through targeting p21 and miR-31 regulated tumor angiogenesis by targeting factor inhibiting hypoxia inducible factor 1, both of which subsequently participated in IBD-associated carcinogenesis.

FUTURE PERSPECTIVE IN IBD DIAGNOSTIC AND TREATMENT

Investigations described above showed that special miRNAs suppressing functional targets played a pro-inflammatory or anti-inflammatory role in regulating the pathogenic mechanism of IBD, including activation of NFκB, increased intestinal epithelial permeability, abnormal autophagic activity and so on. It means inflam-

matory response, intestinal epithelial barrier and other mechanisms involved in IBD can be regulated by targeting miRNAs, indicating the potential of miRNAs as therapeutic targets for IBD. Besides studying the function of IBD-associated miRNAs *in vitro*, some researchers had administrated miRNAs into mice with experimental colitis by different methods to investigate their functional and therapeutic effect *in vivo*. Inhibition of miR-30c and miR-130a in mice by ileal loop assay suppressed AIEC-induced down-regulation of ATG5 and ATG16L1 expression and decreased intestinal inflammation[69]. Overexpression of miR-146b in DSS-induced colitis mouse *via* intraperitoneal injection relieved intestinal inflammation and increased the survival rate of mouse[40]. MiR-141 intracolonic administration in the colon of TNBS-induced and IL-10 KO mice regulated leukocyte infiltration and alleviated intestinal inflammation[62]. These data showed the effective ways to administrate miRNAs into human and the possibilities for the future clinical applications of miRNA-based therapeutic approaches in IBD.

There have been several studies that identified altered miRNA profiles in both serum and inflamed tissue in patients with UC and CD compared with controls, which have been reviewed by Coskun *et al*[76]. Circulating miRNAs in serum exist in membrane vesicles, such as exosomes[77], or form a complex with lipid protein carriers, such as high-density lipoproteins (HDL)[78]. So these circulating miRNAs are protected from blood RNAses and relatively stable compared with mRNA and protein, which make themselves serving as ideal noninvasive blood biomarkers in patients with IBD. In addition, the aberrant expression of miRNAs in inflamed tissues of patients with UC could also help in IBD diagnosis.

CONCLUSION

MiRNAs are a class of potential gene regulators of critical importance in the pathogenesis of IBD. It has been demonstrated that miRNAs have the possibility to be used as biomarkers and therapeutic target in IBD. Although our knowledge about the miRNAs regulation of IBD has considerably advanced over the last several years, multiple areas warrant future investigation. Most studies have focused on one miRNA which targets a single mRNA. One area worth future investigation is a key miRNA targeting multiple mRNAs or several miRNAs combination targeting a key mRNA. The other area worth future investigation focuses on the roles of miRNAs in human studies. Most of our understanding of the functions of miRNAs associated with IBD is based on cell cultures and murine models. Further investigating the roles of miRNAs in the human context will improve our knowledge of miRNAs in the pathogenesis and diagnosis of IBD and pave the way for miRNA-based therapies.

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TOPIC HIGHLIGHT

WJGP 5th Anniversary Special Issues (3): Pancreatitis

Review of the diagnosis, classification and management of autoimmune pancreatitis

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Abstract

Autoimmune pancreatitis (AIP) is a rare form of chronic pancreatitis, with as yet undetermined incidence and prevalence in the general population. Our understanding of it continues to evolve. In the last few years, 2 separate subtypes have been identified: type 1 AIP has been recognised as the pancreatic manifestation of a multiorgan disease, named immunoglobulin G4 (IgG4)-related disease while type 2 AIP is a pancreas specific disorder not associated with IgG4. International criteria for the diagnosis of AIP have been defined: the HISORt criteria from the Mayo clinic, the Japan consensus criteria and, most recently, the international association of pancreatology "International Consensus Diagnostic Criteria". Despite this, in clinical practice it can still be very difficult to confirm the diagnosis and differentiate AIP from a pancreatic cancer. There are no large studies into the long-term prognosis and management of relapses of AIP, and there is even less information at present regarding the Type 2 AIP subtype. Further studies are necessary to clarify the pathogenesis, treatment and long-term outcomes of this disease. Critically for clinicians, making the correct diagnosis and differentiating the disease from pancreatic cancer is of the utmost importance and the greatest challenge.

Key words: Pancreatitis; Autoimmunity; Pancreatic cancer; Autoimmune pancreatitis; Immunoglobulin G4-related disease

Core tip: Type 1 autoimmune pancreatitis (AIP) is the pancreatic manifestation of a multiorgan disease, named immunoglobulin G4 (IgG4)-related disease while type 2 AIP is a pancreas specific disorder not associated with IgG4. Making the correct diagnosis and differentiating the disease from pancreatic cancer is of the utmost importance; an agreed diagnostic pathway should be in place and a multidisciplinary approach taken with each patient.

O'Reilly DA, Malde DJ, Duncan T, Rao M, Filobbos R. Review of the diagnosis, classification and management of autoimmune pancreatitis. *World J Gastrointest Pathophysiol* 2014; 5(2): 71-81 Available from: URL: http://www.wjgnet.com/2150-5330/full/v5/i2/71.htm DOI: http://dx.doi.org/10.4291/wjgp.v5.i2.71

INTRODUCTION

As early as 1961, Sarles *et al*[1] described a form of idiopathic chronic pancreatitis with obstructive jaundice and hypergammaglobulinaemia, with the suspicion that there was an underlying autoimmune process. It was not until

1995, when Yoshida *et al*[2] coined the term "autoimmune pancreatitis" (AIP) that this concept was widely accepted and AIP differentiated from other forms of chronic pancreatitis. Since then, progress has been made in our understanding of the pathophysiology of AIP; type 1 AIP has been recognised as the pancreatic manifestation of a multiorgan disease, named IgG4-related disease, while type 2 AIP is a pancreas specific disorder not associated with IgG4[3,4]. This review gives an overview of current thinking on the pathology of AIP, its clinical features (including serology), classification and treatment. Emphasis is placed upon the diagnostic challenge of distinguishing AIP from pancreatic cancer.

SEARCH STRATEGY

This review of the English language literature on the classification, diagnosis and management of autoimmune pancreatitis is based on papers contained within the PubMed database. Individual searches of the PubMed database were performed with the boolean operator AND, using the terms: "Autoimmune pancreatitis", "Acute pancreatitis", "Chronic pancreatitis", "Pancreatic cancer". The abstracts were screened for eligibility and all relevant publications were requested as full-text articles. References used in requested papers were then checked for any further studies of potential interest.

PATHOPHYSIOLOGY OF AIP

A definitive autoantigen for AIP has not yet been identified. Human leucocyte antigen (HLA) association studies in Japan have reported an association with HLA serotypes DRB1*0405 and DQB1*0401[5]. This was not confirmed in a Korean study but DQβ1-57 without aspartic acid was associated with disease relapse[6]. Single nucleotide polymorphisms identified in association with either disease susceptibility or recurrence include: cytotoxic T-lymphocyte associated antigen 4, tumour necrosis factor-α and Fc receptor-like 3[7]. However, studies of genetic risk factors in AIP remain at an early stage of investigation. A genome-wide association study in AIP would likely advance our understanding significantly.

Potential initiating mechanisms include bacterial infection and molecular mimicry[7]. Substantial homology exists between human carbonic anhydrase Ⅱ and the α-carbonic anhydrase of Helicobacter pylori[8]. In theory, antibodies directed against bacterial components could behave as autoantibodies by means of molecular mimicry in genetically predisposed persons[7]. Thus, autoimmunity is widely regarded as the initial stimulus for the Th2-cell immune response associated with AIP. Antibodies directed against potential autoantigens, such as carbonic anhydrase, lactoferrin, trypsinogen and pancreatic secretory trypsin inhibitor, may give rise to the systemic manifestations of AIP[7-11].

Studies using animal models of experimental autoimmune pancreatitis have significant limitations, as the disease does not occur spontaneously. Current models exhibit considerable variation in target antigens, differing methods for immune staining and differing mouse strains but have provided evidence that the disease is most likely T cell mediated, with highly beneficial effects observed with agents such as the mammalian target of rapamycin (mTOR) inhibitor, sirolimus, which increases the number and activity of regulatory T-cells[4].

SUBTYPES OF AUTOIMMUNE PANCREATITIS

Type 1

This is the more classically described and recognised form of the disease. It is now recognised as a pancreatic manifestation of an immunoglobulin G4 (IgG4) related systemic disease[4,7,12-14]. It is associated with histological findings of a lymphoplasmacytic sclerosing pancreatitis (LPSP). This consists of a dense lymphoplasmacytic infiltration and fibrosis involving the pancreatic lobules, ducts and peripancreatic adipose tissue. Storiform or "swirling" fibrosis and obliterative phlebitis are also characteristic features[15-17]. The lymphoplasmacytic infiltrate is also rich in IgG4 positive cells[18]. It is frequently associated with sclerosing extrapancreatic lesions such as sclerosing cholangitis, retroperitoneal fibrosis and sclerosing sialadenitis[13,19-21]. Type 1 AIP tends to affect older males, with 80% of patients being over 50 years of age at the time of presentation. It is also associated with elevation in serum levels of IgG4 in up to 75% of patients[19,20].

The HISORt criteria from the Mayo clinic[22] and the Japanese consensus criteria[23] were mainly produced to facilitate the diagnosis of Type Ⅰ AIP.

Type 2

This is a relatively recently described form of AIP[3,4]. It has a unique histological pattern, consisting of an idiopathic duct-centric pancreatitis or AIP with a granulocytic epithelial lesion. The inflammation is centred on the exocrine pancreatic system, with neutrophilic infiltration within the lumen and epithelium of the interlobular ducts being a characteristic feature. The neutrophils are sometimes so numerous that microabscesses can be seen in the lobules and ducts. The entire wall of the duct may be infiltrated by neutrophils and plasma cells. The infiltrate frequently involves the duct epithelium and can obliterate it. It differs from LPSP in that there is little obliterative phlebitis and the inflammatory infiltrates have few IgG4 positive cells[24,25].

Much less is known regarding the clinical features of Type 2 AIP. However it appears to be associated with a younger subset of patients and there is no gender preponderance. There also appears to be an association with ulcerative colitis. Type 2 AIP patients usually have a dramatic response to steroid therapy, associated with a low frequency of relapse[25]. Until recently, existing criteria have not been that helpful in the diagnosis of type 2 AIP, but with recent publication of the International Association of Pancreatology (IAP) diagnostic guide-

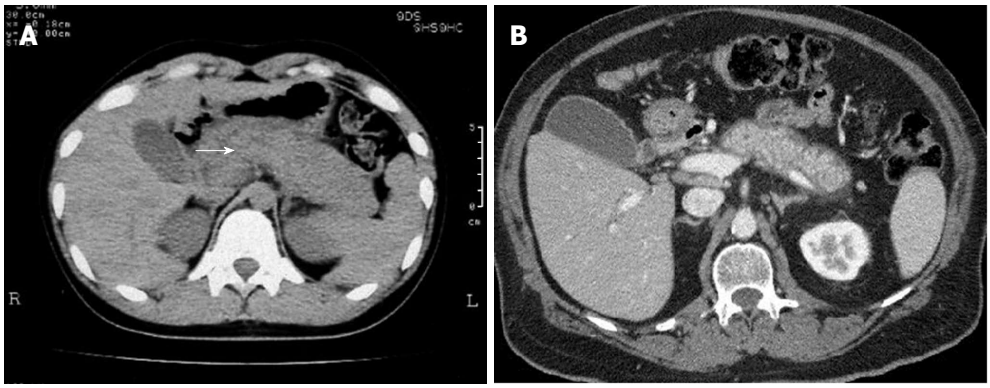

Figure 1 Computed tomography. A: Computed tomography (CT) findings in autoimmune pancreatitis: Showing diffuse enlargement and a "sausage like" appearance of the pancreas (arrow); B: Axial contrast enhanced CT image demonstrating a characteristic low signal rim or halo surrounding the body and tail of the pancreas in another patient with autoimmune pancreatitis.

lines[26], it is anticipated that more data will confirm and further characterise this subtype.

Variation in the geographic distribution of the two subtypes may help to explain the heterogeneity of disease morphology observed worldwide.

CLINICAL PRESENTATION

The presentation of AIP is varied, but a classical picture is obstructive jaundice, often painless or with mild epigastric pain. Less commonly, new onset diabetes or symptoms of pancreatic insufficiency and weight loss may occur. A rarer presentation is acute pancreatitis and its sequelae. A characteristic feature of type 1 AIP is extrapancreatic other organ involvement. In Type 1 AIP the majority are male and over the age of 50. Some patients are only diagnosed post-operatively, having had a resection for a presumed pancreatic cancer.

The clinical picture in Type 2 autoimmune pancreatitis appears to affect a younger cohort of patients, more likely in their 4th decade of life and there is no gender preponderance. There are more reports of this group presenting with acute pancreatitis, and a higher frequency of association with ulcerative colitis[25]. However, the numbers of patients reported in the worldwide literature are still very small and further clarity is expected to emerge with time, to further define this subgroup.

SEROLOGY

Type 1 AIP is associated with a number of serological abnormalities, in particular an elevated IgG4[18,19]. Hamano *et al*[19] reported that a cut-off value of 135 mg/dL for serum IgG4 concentration differentiates AIP from pancreatic cancer with an accuracy of 97%, a sensitivity of 95% and specificity of 97%. An elevated IgG4 is however not diagnostic of Type 1 AIP, but is a characteristic along with other identified criteria. The Mayo clinic reported a sensitivity, specificity and positive predictive value of 76%, 93% and 36% respectively, using a cut-off value for IgG4 of 140 mg/dL[27]. Elevated IgG4 levels also may be found in PSC, acute and chronic pancreatitis and up to 10% of patients with pancreatic cancer[19]. Serum IgG4 of more than 2 times the upper limit of normal greatly increases the specificity for AIP.

Other elevated markers may include: rheumatoid factor, carbonic anhydrase, antilactoferrin and antinuclear antibodies[9,10]. A study from Frulloni *et al*[28] in Italy identified an anti plasminogen-binding peptide antibody which was elevated in 94% of their AIP patients. In this cohort of AIP patients, they had a relatively low prevalence of elevated IgG4 (at only 54%). This was a single centre study of 20 patients and clearly more studies are needed to assess this and other autoantibodies as potential markers for AIP and as aids to distinguish AIP from pancreatic malignancy.

IMAGING

Imaging is essential in establishing a diagnosis of AIP. Three different forms of the disease process can be seen, including diffuse, focal or multifocal disease, with the diffuse form being the most common. A contrast enhanced computed tomography (CT) scan is the gold standard for investigation as it is essential to look for a pancreatic malignancy and evidence of metastatic disease. Figure 1A shows the contrast enhanced CT findings characteristic of Type 1 AIP: a diffusely enlarged or "sausage shaped" pancreas with loss of the normal pancreatic clefts and delayed and peripheral rim enhancement[29]. Figure 1B shows a characteristic surrounding hypoattenuating/low signal rim or halo on CT. Generally there is minimal associated peripancreatic soft tissue stranding and rarely inflammation of the mesentery. Local peripancreatic lymphadenopathy can be observed. Pancreatic calcification and pseudocyst formation is not a recognised typical finding in autoimmune pancreatitis. CT may also find extra pancreatic lesions such as retroperitoneal fibrosis.

The focal form of the disease is less common and is characterized by a focal mass lesion within the pancreas and can be mistaken for pancreatic malignancy (Figure 2). Normally dilatation of the pancreatic duct is less marked in autoimmune pancreatitis than that associated with pancreatic malignancy. Typically the main pancre-

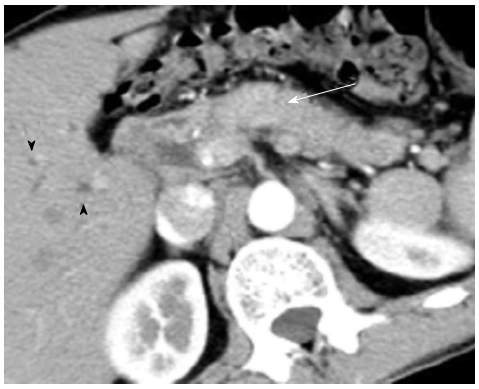

Figure 2 Focal enlargement of the pancreatic parenchyma in the head of the pancreas (arrow), and dilatation of the intrahepatic bile ducts visible (arrowheads).

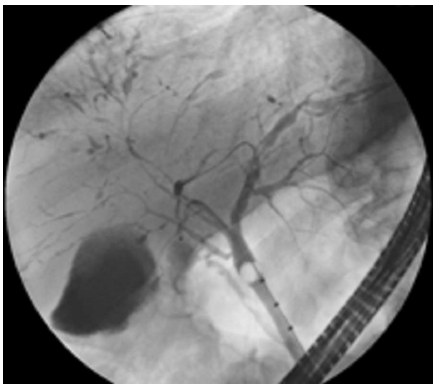

Figure 3 Endoscopic retrograde cholangiopancreatography findings of multiple and focal strictures and dilatation in the intrahepatic bile ducts in autoimmune pancreatitis.

atic duct is irregularly narrowed in affected segments of the pancreas. In the multifocal form of the disease, the pancreatic duct is of normal calibre in non affected segments. Magnetic resonance imaging (MRI) shows diffuse or localised enlargement of the pancreas with lower density in T1 weighted images and higher density in T2 weighted images compared with each of the liver images.

Sclerosing cholangitis is observed in a proportion of patients with autoimmune pancreatitis and can be seen in isolation. The intrapancreatic portion of the common bile duct is the most affected segment of the biliary tree. Affected segments of the biliary tree demonstrate irregular stricturing and associated contrast enhancement. Generally strictures associated with autoimmune disease are long and continuous whereas multifocal short strictures are more typical of primary sclerosing cholangitis (PSC), although differentiation between the two can be difficult in some cases (Figure 3).

Endoscopic ultrasound (EUS) is being used more frequently for pancreatic core biopsies, which acts as an aide to histological diagnosis and is likely superior to fine needle aspiration (FNA)[30]. Typical EUS findings in AIP include: diffuse hypoechoic spots, absence of a discreet mass and chronic inflammatory cells on aspiration cytology. Mizuno *et al*[30] and Levy *et al*[31] have demonstrated the benefits of the use of EUS-guided biopsies to aid in the diagnosis of AIP[32]. Future refinement of diagnosis may be obtained with the use of contrast-enhanced EUS and elastography[4]. The use of positron emission tomography (PET) and its potential role for diagnosis of AIP is yet to be validated[33].

OTHER ORGAN INVOLVEMENT

In Type 1 AIP, which may be considered part of an IgG4 systemic disease process, there are a significant number of associated extrapancreatic lesions. The most common are: hilar lymphadenopathy, sclerosing cholangitis, retroperitoneal fibrosis, salivary and lacrimal gland involvement and tubulointerstitial nephritis[21,22,34-37]. There are other conditions that have been less frequently reported, such as hypophysitis and chronic thyroiditis. It is this link to other organ involvement that led clinicians to consider AIP as part of a systemic IgG4 related disease, analogous to sarcoidosis, another systemic disease in which diverse organ manifestations are linked by the same histopathological characteristics[7].

Biliary disease is one of the most common extrapancreatic manifestations of AIP. Although the main cause of jaundice in AIP is obstruction at the level of the intrapancreatic portion of the common bile duct, associated with an inflammatory pancreatic head mass, stricturing in the rest of the biliary tree is increasingly recognised. This condition has been termed IgG4-associated cholangitis (IAC) and has been reported to occur in 20%-88% of cases of AIP[38]. A possible overlap between IAC and PSC is also suggested by the finding that 9%-36% of patients with PSC have increased serum IgG4 levels, compared with less than 1% in other liver diseases[39,40]. Of note, PSC patients with raised serum IgG4 levels have a more rapid progression to liver transplantation compared to those with normal levels[38].

Extrapancreatic disease can be a useful factor in the diagnosis of autoimmune pancreatitis, distinguishing it from pancreatic cancer, and forms part of the HISORt criteria. It also provides collateral evidence for AIP, according to the IAP diagnostic guidelines. The evidence to support the association between these conditions and AIP include: multiple reports indicating frequent or intimate concurrence, extrapancreatic pathological findings of severe lymphoplastic infiltration and storiform fibrosis with numerous IgG4 positive plasma cell infiltrations and a combined favourable response to steroid therapy[23,26,41].

DIAGNOSIS OF AIP

There is no single diagnostic test for AIP and there is significant variation in clinical practice worldwide, particularly between Asia and North America/Europe. The biggest challenge associated with the diagnosis of AIP is that it can closely resemble pancreatic cancer. Most commonly AIP presents with obstructive jaundice and pancreatic enlargement; other worrying symptoms such as

Table 1 The Mayo clinic HISORt criteria for the diagnosis of autoimmune pancreatitis

Category	Criteria
Histology	One of the following: Periductal lymphoplasmacytic infiltrate with obliterative phlebitis and storiform fibrosis (LPSP) Lymphoplasmacytic infiltrate with storiform fibrosis showing abundant IgG4 positive cells (> 10 cells/HPF)
Imaging (CT)/(MRI)	Typical; diffusely enlarged gland with diffuse rim enhancement, diffusely irregular attenuated pancreatic duct Other; focal pancreatic mass or enlargement; focal pancreatic duct stricture; pancreatic duct stricture, pancreatic atrophy; pancreatic calcification or pancreatitis
Serology	Elevated serum IgG4 level
Other organ involvement	Hilar/intrahepatic biliary strictures, persistent distal biliary strictures, parotid or lacrimal gland involvement, mediastinal lymphadenopathy or retroperitoneal fibrosis
Response to steroid therapy	Resolution/Marked improvement of pancreatic or extrapancreatic manifestion with steroid therapy

LPSP: Lymphoplasmacytic sclerosing pancreatitis; CT: Computed tomography; MRI: Magnetic resonance imaging; IgG4: Immunoglobulin G4; HPF: High powered field.

weight loss and new onset diabetes may also be present. Less commonly AIP can present with features of acute pancreatitis or unexplained pancreatic insufficiency. Misdiagnosis at this stage has the potential to be catastrophic, as an undiagnosed cancer may cause delay or loss of the opportunity for potential curative cancer surgery. The opposite scenario of a pancreatoduodenectomy being undertaken for benign disease (with its high risk of morbidity and mortality) is also unsatisfactory.

In 2002 the Japan Pancreas Society published guidelines for diagnosis of AIP. These were updated in 2006 and again in 2009. The HISORt criteria from the Mayo[22] clinic require histology, imaging, serology, other organ involvement and response to therapy for diagnosis. The inclusion of response to steroids as part of the diagnosis is one of the criteria that differentiates the Mayo recommendations from the Japanese. In Japan, endoscopic retrograde pancreatography (ERP) is routinely performed to aid in the diagnosis of AIP. More recently, the IAP has published their International consensus diagnostic criteria (ICDC)[26], in an attempt to bridge the divide in clinical practise around the globe and offers criteria for the diagnosis of both subtypes of AIP.

SUMMARY OF DIAGNOSTIC CRITERIA

Guidelines regarding diagnostic criteria vary worldwide. Although criteria have been developed by other groups, the most influential come from the United States[22], Japan[23] and the International Association of Pancreatology[26]. Below are the definitions from these three different groups.

Japan/Asian

In 2002 the Japan Pancreas society published their data for the diagnosis of AIP; this was further revised in 2006. In 2009 Okazaki *et al*[23] published the Japanese consensus guidelines for management of autoimmune pancreatitis. There are 3 main criteria. For the diagnosis to be confirmed, criterion 1 must be present along with criterion 2 and/or criterion 3.

Imaging: Diffuse or segmental narrowing of the main pancreatic duct with irregular wall and diffuse or segmental enlargement of the pancreas with imaging studies such as: Ultrasound, CT, MRI or ERP.

Serology: High serum gammaglobulin IgG or IgG4, or the presence of autoantibodies, such as antinuclear antibodies or rheumatoid factor.

Histology: Marked inter-lobular fibrosis and prominent infiltration of lymphocytes and plasma cells in the periductal area, occasionally with lymphoid follicles in the pancreas.

There is an optional criterion for patients fulfilling criterion 1 alone: a response to steroid therapy, with the caveat that malignancy of the pancreas or biliary tract must be excluded. In 2006, a mandatory ERP became part of these guidelines.

United States

The Mayo clinic HISORt criteria are based on 5 main diagnostic criteria: histological findings, imaging, serology, other organ involvement and response to steroid therapy[22,42]. The detailed features are listed in Table 1. Essentially, use of these criteria enable patients to be categorised into three diagnostic groups [diagnostic pancreatic histology, typical imaging and serology, steroid responders (after careful work-up to exclude cancer)]. Patients in one or more of these categories are deemed to have AIP.

International association of pancreatology

The goals of the IAP were to develop international consensus on the diagnostic criteria that can be applied worldwide, to safely diagnose AIP and to avoid a misdiagnosis of pancreatic cancer[26]. They reviewed all existing criteria, including the Japanese and HISORt. The consensus opinion was that the terms type 1 and type 2 should be used to describe the clinical profiles associated with LPSP and idiopathic duct-centric pancreatitis, respectively. Tables 2-4 shows the diagnostic criteria for definitive and probable AIP type 1 and 2. This uses a combination of 1 or more of 5 cardinal features of AIP: (1) imaging features of the following: pancreatic parenchyma (on

Table 2 International consensus diagnostic criteria for type 1 autoimmune pancreatitis

	Diagnosis of type 1 AIP		
Diagnosis	**Cardinal feature**	**Imaging evidence**	**Collateral evidence**
Definitive type 1	Histology	Typical/indeterminate	Confirmed LPSP
	Imaging	Typical Inderminate	Any level 1/2 ⩾ 2 level 1
	Steroid response	Indeterminate	Level 1 S/OOI and Rt OR Level 1 D and level 2 S/OOI/H and Rt
Probable type 1		Indeterminate	Level 2 S/OOI/H and Rt

LPSP: Lymphoplasmacytic sclerosing pancreatitis; AIP: Autoimmune pancreatitis; S: Serology; OOI: Other organ involvement; Rt: Response to steroid therapy; H: Histology

CT/MRI) and pancreatic duct [ERCP or magnetic resonance cholangiopancreatography (MRCP)]; (2) serology (IgG, IgG4 and antinuclear antibody); (3) other organ involvement (OOI); (4) histopathology of the pancreas; and (5) response to steroid therapy.

Level 1 and level 2 criteria are then specified, according to the strength that specific findings add to the likelihood of diagnosis. For example, a greater than 2-fold elevation of IgG4 is considered a level 1 criteria; a lesser elevation level 2. Further specification is given for pancreatic ductal and parenchymal appearances, histology and response to steroids. Thus, definite and probable type 1 and type 2 AIP can be diagnosed.

In all cases the criteria are geared towards excluding a diagnosis of pancreatic cancer rather than screening for AIP, *i.e.,* they emphasise specificity rather than sensitivity. Only the IAP guidelines include the diagnostic features of Type 2 autoimmune pancreatitis.

DISTINGUISHING AIP FROM PANCREATIC CANCER

In view of its presentation with obstructive jaundice and pancreatic enlargement, AIP often needs to be distinguished from pancreatic cancer. As ERCP features have been reported to have limited sensitivity to diagnose AIP in Western centres, Figure 4 shows a strategy to aid in differentiation, diagnosis and management of AIP versus pancreatic cancer, based upon the experience and algorithm of the Mayo Clinic[22]. When features highly suggestive of either AIP or pancreatic cancer are present (a low-density mass, pancreatic ductal dilatation, pancreatic duct cut off, upstream pancreatic atrophy or liver lesions suggestive of metastases), the diagnostic and management pathway is usually clear. However, in indeterminate cases, further cancer work-up is required in the first instance. In the event of a negative cancer work-up, a pancreatic core biopsy is helpful in categorising patients if a positive diagnosis can be made. Equivocal or inadequate results are more problematic and a trial of steroids or surgery should be considered.

Table 3 International consensus diagnostic criteria for type 2 autoimmune pancreatitis

	Diagnosis of type 2 AIP	
Diagnosis	**Imaging evidence**	**Collateral evidence**
Definitive type 2	Typical/indeterminate	Histologically confirmed or clinical inflammatory bowel disease and level 2H and Rt
Probable type 2	Typical/indeterminate	Level 2 H/clinical inflammatory bowel disease and Rt

AIP: Autoimmune pancreatitis; Rt: Response to steroid therapy; H: Histology.

Using the Mayo Clinic strategy, AIP was successfully distinguished from pancreatic cancer in most patients but 27% required a pancreatic core biopsy, steroid trial or surgery to clarify the diagnosis[43]. Kamisawa *et al*[44] have reported their Japanese strategy when investigating patients presenting with mass lesions. Strategies based upon the Japanese criteria can be simpler but rely on ERP. Despite this, surgery was still required to make a diagnosis in 6 of 37 (16%) patients. Further evaluation and comparison is required to determine the optimal and least invasive diagnostic pathway.

In our view, when distinguishing AIP from pancreatic cancer, the most important tips or principals of diagnosis include the following: (1) clinical presentations not suggestive of AIP include marked cachexia, anorexia and severe pain requiring opiates; (2) a thorough negative work up for other aetiologies should be undertaken, in particular for pancreatic or biliary cancer; (3) histological diagnosis of AIP requires preservation of tissue architecture (showing lymphoplasmacytic infiltrate with >10 IgG4 positive cells/high power field), which renders FNA less helpful for diagnosis; (4) steroid therapy should only be commenced when other aetiologies for pancreatic disease have been excluded, and only in those patients whose response may be adequately assessed. It should not be used as a substitute for a thorough search for the aetiology; (5) objective improvement in the appearance of the pancreas on cross-sectional imaging should be evident within 2 wk of steroid use. Subjective improvement in symptoms or even a decline in serum IgG4 levels can occur in pancreatic cancer or lymphoma and should not be used as response criteria; (6) in AIP, CA 19-9 levels drop with treatment; a rising CA 19-9 suggests this diagnosis is incorrect; and (7) the diagnosis of AIP is difficult. An agreed diagnostic pathway should be in place and a multidisciplinary approach taken with each patient, to ensure that pancreatic cancer patients are not treated with steroids and, conversely, AIP patients not treated with cancer surgery.

INITIAL TREATMENT, MAINTENANCE AND RELAPSE

Although it is well established that spontaneous resolution can occur in up to 30% of cases of AIP[45], symptomatic patients are best treated with corticosteroids (*i.e.*,

Table 4 International consensus diagnostic criteria level 1 and 2 criteria for type 1 and 2 autoimmune pancreatitis

	Type 1 AIP	
Criterion	**Level 1**	**Level 2**
Parenchymal imaging	Typical: Diffuse enlargement with delayed enhancement	Indeterminate: Focal enlargement with delayed enhancement
Ductal imaging (ERP)	Long or multiple strictures (> 1/3 duct length) without upstream dilatation	Focal narrowing without upstream dilatation (< 5 mm)
Serology	IgG4 > 2x upper limit	IgG4 1-2x upper limit
Other organ involvement	Extrapancreatic organ histology. Any 3 of : 1 Lymphoplasmacytic infiltration with fibrosis and without granulocytic infiltration 2 Storiform fibrosis 3 Obliterative phlebitis 4 > 10 cells/HPF IgG4-positive cells Typical radiology. Any one of: 1 Segmental/multiple proximal or distal biliary stricture 2 Retroperitoneal fibrosis	Extrapancreatic organ histology including bile duct biopsies. Both of: 1 Marked lymphoplasmacytic infiltration without granulocytic infiltration 2 10 cells/HPF IgG4-positive cells Physical or radiological evidence of at least one of: 1 Enlarged salivary/lachrymal glands 2 Renal involvement
Histology of pancreas	LPSP and 3 of: 1 Periductal lymphoplasmacytic infiltrate without granulocytic infiltration 2 Obliterative phlebitis 3 Storiform fibrosis 4 > 10 cells/HPF IgG4-positive cells	LPSP and 2 of: 1 Periductal lymphoplasmacytic infiltrate without granulocytic infiltration 2 Obliterative phlebitis 3 Storiform fibrosis 4 > 10 cells/HPF IgG4-positive cells
Response to steroid (Rt)	Rapid (< 2 wk) radiological demonstration of marked improvement in pancreatic/extrapancreatic manifestations	
Type 2 AIP		
Parenchymal imaging	Typical: Diffuse enlargement with delayed enhancement	Indeterminate: Focal enlargement with delayed enhancement
Ductal Imaging (ERCP)	Long (> 1/3 duct length) or multiple strictures without upstream dilatation	Focal narrowing without marked upstream dilatation (< 5 mm)
Other organ involvement		Clinically diagnosed inflammatory bowel disease
Histology of pancreas	IDCP. Both of: 1 Granulocytic infiltration of duct wall with or without acinar inflammation 2 0-10 cells/HPF IgG4-positive cells	Both of : 1 Granulocytic and lymphoplasmacytic acinar infiltrate 2 0-10 cells/HPF IgG4-positive cells
Response to steroid (Rt)	Rapid (< 2 wk) radiological demonstration of marked improvement in manifestations	

LPSP: Lymphoplasmacytic sclerosing pancreatitis; IDCP: Idiopathic duct-centric pancreatitis; AIP: Autoimmune pancreatitis; IgG4: immunoglobulin G4; ERP: Endoscopic retrograde pancreatography; Rt: Response to steroid therapy; HPF: High powered field.

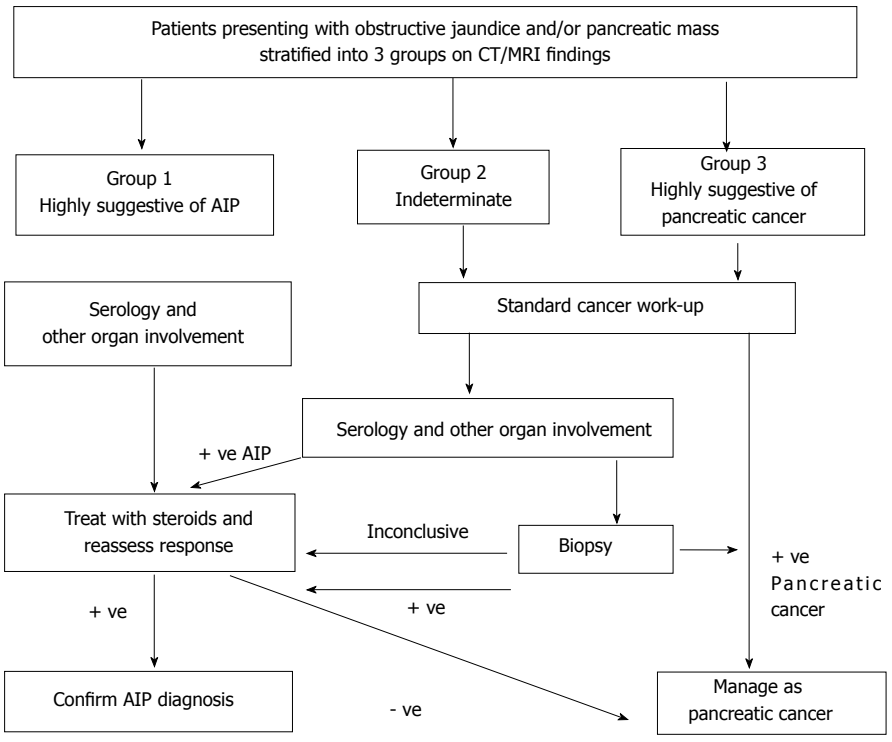

Figure 4 A strategy for distinguishing autoimmune pancreatitis from pancreatic cancer (based upon the Mayo clinic strategy[23]). CT: Computed tomography; MRI: Magnetic resonance imaging; AIP: Autoimmune pancreatitis.

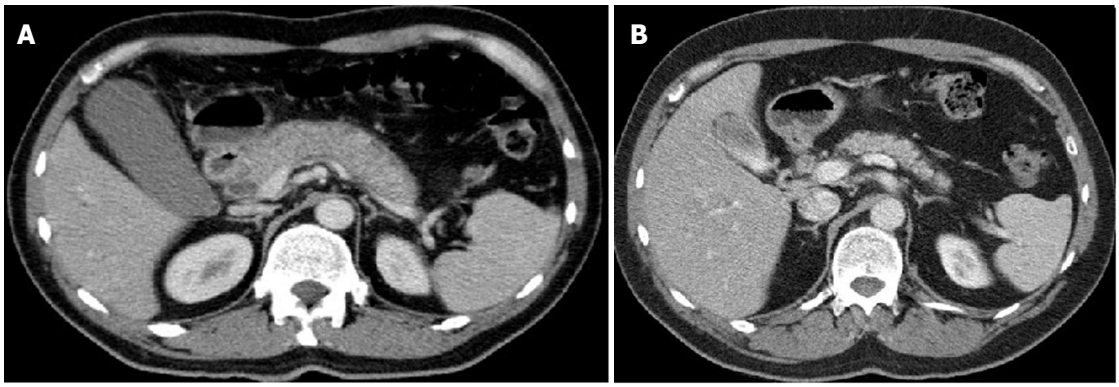

Figure 5 Axial computed tomography image. A: Demonstrating a characteristic sausage shaped enlarged pancreas with surrounding halo in keeping with autoimmune pancreatitis; B: From the same patient 8 mo later following corticosteroid therapy demonstrating response to treatment.

prednisolone). A large multicentre retrospective trial from Kamisawa *et al*[46] in 2009 identified 563 patients with AIP and found that 98% responded to steroid therapy versus 74% that improved without. The response can be dramatic. An improvement of imaging findings, with resolution of pancreatic enlargement and biliary stricturing can be seen following corticosteroid treatment in Figure 5.

Initial steroid dose varies slightly according to guideline. In the Mayo clinic a standard initial dose is 40 mg per day of oral prednisolone, for 4 wk. If there is obvious clinical and radiological improvement, the dose is decreased by 5 mg/wk until it is stopped at 11 wk[47]. The Japanese consensus statement on treatment and prognosis of AIP specifies that an initial oral prednisolone dose for induction of remission of 0.6 mg/kg per day is recommended. The initial dose is administered for 2-4 wk and then gradually tapered. The IAP guidelines specify dose of prednisolone of 0.6-1.0 mg/kg per day with reassessment at 2 wk[26]. The study that formed the basis of the IAP consensus guideline regarding the two week reassessment after a trial of steroid treatment was the prospective study of Moon *et al*[40]. After a 2-wk steroid trial, response to steroids was assessed on the basis of a marked improvement in pancreatic duct narrowing, and a reduction in size of the pancreatic mass. All patients who responded to steroids (15/22) were diagnosed as AIP after a median follow-up of 27 mo, whereas all patients who did not respond to steroids (7/22) were diagnosed with pancreatic cancer, with a complete resection being possible in 6/6 patients who accepted surgery. Induction of remission with rituximab, a monoclonal antibody directed against the CD20 antigen on B lymphocytes, is currently under investigation[4, 48].

Differing rates of tapering are also recommended. Chiefly, the distinction is between the 5 mg/wk reduction of prednisolone, after initial treatment versus a more gradual approach recommended by the Japanese. The Japanese consensus document advocates that the dose be tapered by 5 mg every 1-2 wk, after 2-4 wk at the initial dose, based on changes in the clinical manifestations, biochemical blood tests (such as liver enzymes and IgG or IgG4 levels), and repeated imaging findings (US, CT, MRCP, ERCP). The dose is tapered to a maintenance dose over a period of 2-3 mo.

A maintenance dose of 2.5-5.0 mg/d is recommended by the Japanese, to prevent relapse. This is not recommended by the Mayo clinic group, who take the view that the universal use of maintenance therapy is not warranted because the risks of long term steroid use outweighs the benefits[47]. A wide range of relapse rates are reported, from 22%-100%[38]. In the Mayo clinic experience of 78 type 1 AIP patients with a median follow-up of 42 mo, symptomatic disease relapse was seen in 47% patients with a 3-year cumulative relapse rate of 59% in type 1 AIP patients who were medically managed[49]. This wide variation in relapse rates may be due to lack of a uniform definition of disease relapse, short follow-up times, small patient populations, differences in steroid treatment regimens, lack of identification of subtypes and ethnic variation.

Treatment of relapse is effectively achieved with corticosteroids. The Japanese consensus guideline states that remission can be obtained with the same prednisolone dose as the initial dose in most relapsed AIP cases, but that it may be necessary to taper more gradually[50]. In Europe and the United States, azathioprine has often been introduced for the treatment of relapsing disease, despite pancreatitis being a known side-effect of azathioprine. Acute pancreatitis occurs in approximately 2% of cases of azathioprine use, but there is no evidence as yet that this risk is increased in AIP. Some advocate that, as in autoimmune hepatitis (AIH), AIP should be managed by azathioprine, with or without low dose steroids for at least three years. This analogy is not completely convincing; in AIH disease relapse is almost universal in those who cease immunosuppression early whereas the relapse rate is much more variable in AIP. Moreover, in a recent study from the Mayo group, in patients with relapsing AIP, azathioprine was not shown to be superior to another course of steroids alone[51].

Related areas of management include: biliary stenting, treatment of endocrine and exocrine failure and consideration of pancreatic cancer risk in AIP. Patients presenting with obstructive jaundice should certainly be considered for biliary stenting at ERCP. This is the Japanese practice[50] as it fits in with their strategy, which

includes endoscopic pancreatography in an intrinsic role among their diagnostic tests. However, resolution of jaundice occurs in AIP with steroid treatment without stenting, and obviously, this avoids the risks of ERCP. Avoiding the morbidity and mortality associated with ERCP and biliary stenting is also increasingly attempted in suspected pancreatic cancer, as routine preoperative biliary drainage in patients undergoing surgery for cancer of the pancreatic head increases the rate of overall complications[52]. Diabetes mellitus is common in AIP and although improvement has been reported upon commencing steroids, often requires treatment with oral hypoglycemic agents or insulin[47]. Similar considerations apply to exocrine pancreatic failure. Patients should receive pancreatic enzyme supplementation if pancreatic exocrine insufficiency is suspected, based on the presence of clinical features such as: diarrhoea, steatorrhoea, weight loss, metabolic bone disease or vitamin or mineral deficiency. There is no established association between AIP and pancreatic cancer, just case reports of both conditions. It is not unreasonable to suppose the AIP shares a similar association with pancreatic cancer as with other forms of chronic pancreatitis, given the florid inflammatory response that may persist and relapse over years. Careful follow up of these patients will provide the definitive answer to this question but in the interim this seems the prudent approach to take.

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Alcoholic pancreatitis: A tale of spirits and bacteria

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Abstract

Alcohol is a major cause of chronic pancreatitis. About 5% of alcoholics will ever suffer from pancreatitis, suggesting that additional co-factors are required to trigger an overt disease. Experimental work has implicated lipopolysaccharide, from gut-derived bacteria, as a potential co-factor of alcoholic pancreatitis. This review discusses the effects of alcohol on the gut flora, the gut barrier, the liver-and the pancreas and proposes potential interventional strategies. A better understanding of the interaction between the gut, the liver and the pancreas may provide valuable insight into the pathophysiology of alcoholic pancreatitis.

Key words: Alcohol; Pancreatitis; Fibrosis; Bacteria; Endotoxin; Lipopolysaccharide

Core tip: There is now clear clinical and experimental evidence that bacteria and bacterial products (such as endotoxin) are associated with complications of pancreatitis. Furthermore, results of animal studies support the concept that bacterial endotoxin is an important factor in the initiation and progression of alcoholic pancreatitis.

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INTRODUCTION

Chronic alcohol consumption is a known cause of injury to several organs, most commonly the liver and the pancreas, but also to the heart, lungs and brain. However, it is well understood that only a minority of alcoholics will ever develop clinically overt pancreatic or liver damage and even fewer numbers will develop clinically overt disease in both organs simultaneously although subclinical damage to both organs has been reported to coexist[1]. The fact that only some alcoholics appear to be susceptible to clinical pancreatitis or hepatitis has led to a concerted search for additional trigger/initiating factors for alcohol-induced organ damage.

Over the past two decades clinical and experimental studies have demonstrated that endotoxin lipopolysaccharide (LPS), from the bacterial wall of gram negative bacteria of the human gut, plays a central role in the initiation and progression of alcoholic liver disease[2]. This was initially based on clinical observations of elevated plasma endotoxin concentrations in alcoholics with and without liver disease[3,4]. Experimental evidence in support of the association of endotoxin and liver disease in humans was subsequently provided by animal studies demonstrating that alcohol-fed rats challenged with LPS developed hepatic lesions resembling alcoholic hepatitis in humans[5,6].

Conversely, targeted disruption of the LPS receptor toll like receptor 4 (TLR4) in alcohol-fed animals protected against liver injury[7].

Reports of increased endotoxinemia in pancreatitis emerged a decade later. Several studies have linked the degree of endotoxinemia to the severity and prognosis of acute pancreatitis, regardless of its aetiology[8,9] and the impact of endotoxinemia on multiple organ system failure, in particular pancreatitis-associated lung disease has been corroborated by animal studies[10]. However, it remained elusive whether endotoxinemia was a cause or a consequence of pancreatitis, or both. It has only recently been shown that endotoxin initiates pancreatic necro-inflammation in alcohol-fed rodents[11,12] and promotes pancreatic fibrosis[12].

In healthy subjects, small amounts of endotoxin translocate from the gut lumen to the bloodstream and are naturally cleared by the reticulo-endothelial system. Under the influence of alcohol, bacteria proliferate in the small intestine[13,14], intestinal permeability is increased[15,16], while endotoxin clearance by the reticulo-endothelial system-in particular Kupffer cells in the liver - is diminished[17]. As a result, excess endotoxin is available in the blood stream and exerts its harmful effects on various organs.

This review aims to summarise the mechanisms underlying increased endotoxinemia in alcoholics, describes the role of endotoxin both as an initiating and aggravating factor of pancreatitis and attempts to define a role for the liver as a mediator in pancreatic end-organ damage.

ALCOHOL AND THE GUT FLORA

A human being harbours up to 500 different bacterial species[18], the overall bacterial cell count being 10 times more abundant than the number of eukaryotic cells in the body[19]. The combination of species-which is established during the first year of life and shaped by host genotype[20] as well as dietary factors-varies from individual to individual[21]. Moreover, there is evidence indicating that certain strains of bacteria may be unique to their host[22]. Bacterial concentrations are lowest in the upper gastrointestinal tract due to gastric acid, biliary and pancreatic secretion while the highest density of bacteria is found in the colon. In healthy humans, the gut flora prevents the growth of potential injurious bacteria[18,23], exerts metabolic activities such as the fermentation of non-digestible carbohydrates[24] or vitamin synthesis[25] and plays a role in intestinal cell growth and differentiation[26]. Several factors may influence bacterial luminal content. These include altered gut motility[27], drugs, in particular antibiotics[28] and dietary factors such as alcohol.

Alcohol has been shown to alter the jejunal microflora, since almost 50% of alcoholics with documented recent ethanol abuse displayed an increase in total number of bacteria most of which originated from the faecal flora[13]. These data were confirmed in duodenal juice samples obtained by oesogastroduodenoscopy[14] as well as H_2-breath tests, as a surrogate marker of bacterial proliferation in the proximal gut, in alcoholic subjects[29]. The mechanisms underlying bacterial overgrowth in alcoholism are unknown, but reduction of orocaecal transit time observed in chronic alcoholics[30,31] may offer a partial explanation. It is noteworthy, that alcohol gavage in rodents for 10 wk has the capacity to alter the composition of colonic bacteria[32].

Interestingly, certain bacteria of the gut flora have the capacity to metabolise alcohol to acetaldehyde[33,34]. In alcohol-fed rats, ethanol metabolism by colonic bacteria could be suppressed by ciprofloxacin[35] or a combination of ampicillin and neomycin[36]. In a similar animal model, administration of metronidazole increased alcohol dehydrogenase-containing bacteria and hence colonic acetaldehyde content[37]. While acetaldehyde has been measured in the rodent colon[36] and human gut bacteria have the capacity to metabolise ethanol, there is, to date, no report on acetaldehyde content of the human colon in alcoholics. Nonetheless, the above studies suggest that it would not be unreasonable to implicate acetaldehyde, as the compound that mediates most of the toxic effects of ethanol.

ALCOHOL AND GUT PERMEABILITY

In order for bacteria or bacterial products such as endotoxin to pass into the bloodstream and exert their systemic effects, they are required to cross the gut barrier. In its physiological state, the gut represents an effective barrier, made of a single continuous cell layer from the stomach to the rectum. The cells are sealed together by two sets of highly complex junctions, the more apical tight junction and the adherens junction. Physiologically, tight junctions may allow the passage of small molecules up to a molecular weight of 2000 Da but prevents the translocation of larger molecules, in particular bacterial products or bacteria[38]. In addition to this mechanical barrier, passage of bacteria or bacterial products is prevented by mucus, immunoglobulins, defensins and other antimicrobial products produced by the gut.

Intestinal permeability can be measured non-invasively using oral probes such as ethylene glycol polymers of varying molecular sizes, oligosaccharides (*e.g.*, lactulose), monosaccharides (mannitol) and radiolabeled chelates such as chromium-ethylenediaminetetraacetic acid (Cr-EDTA). All these compounds are poorly absorbed by the normal bowel mucosa and display absent or negligible metabolism. Hence, increased urinary excretion correlates with increased intestinal permeability. It is now acknowledged that the probes are absorbed *via* the paracellular route, implying that competence of the gut barrier depends on the integrity of intercellular junctions[39,40].

Several studies have addressed the question whether alcohol increases gut permeability. Early studies with rats chronically administered alcohol revealed increased permeability to macromolecules such as hemoglobin with a known molecular weight of 17 kDa[41] and horseradish peroxidase with a molecular weight of 44 kDa[42]. Permeability to smaller molecules also appears to be increased in rodents upon ethanol administration as exemplified by

increased lactulose/mannitol ratio. Increased absorption of ^{51}Cr-EDTA, a small molecule of 340 Da, was also observed in chronic alcoholics[42]. An increase in absorption of a molecule of similar size (PEG 400) was reported when alcohol was administered to volunteers with no history of chronic ethanol abuse[16]. The latter data failed to be confirmed by Parlesak *et al*[43] who did not observe a difference in the absorption of polyethylen glycol (PEG) 400 when chronic alcoholics were compared to healthy subjects. In the same study, however, permeability to larger molecules of polyethylene glycol (PEG 1500, 4000 and 10000) was significantly enhanced and the permeability to PEG 10000 in particular was 10-fold higher in alcoholics. Taken together there is experimental and clinical evidence that gut permeability is enhanced by acute and chronic ethanol administration. Permeability seems to be increased for molecules of higher molecular weight (from 1000 Da to at least 44 kDa), which is of particular relevance to the translocation of gut derived bacterial endotoxin, a large compound with a known molecular weight of 40 kDa, as a putative initiating and aggravating factor of alcohol-induced organ damage.

In order to explain increased gut permeability by alcohol, various morphological and molecular studies have been undertaken. There is evidence that alcohol exerts direct toxic effects on the gut mucosa. In an observational study by Gottfried *et al*[44], seven alcoholic subjects with a previously unremarkable oesogastroduodenoscopy were administered 1 g/kg body weight alcohol (35% w/v). Biopsy specimens taken during oesogastroduodenoscopy performed 3 h after alcohol exposure demonstrated transient focal subepithelial hemorrhage which disappeared within 3 d. These observations were corroborated by experimental data in rodents and dogs[45,46]. Studies of histological alterations in patients chronically abusing alcohol have yielded conflicting results since both histological alterations and normal mucosal structure have been described[47]. This may be related to the fact that alcohol-induced mucosal lesions are short-lived due to rapid regeneration of epithelial cells (in the study reporting normal mucosal structure, endoscopies were performed 3-14 d after alcohol withdrawal). At the molecular level, different effects of ethanol on interepithelial junctions in the gut have been described.

Ethanol at high doses has been reported to lead to increased gut permeability via direct action on tight junctions. Ma *et al*[48] measured epithelial resistance and paracellular permeability of the human adenocarcinoma cell line Caco-2 exposed to ethanol. At ethanol concentration ranging from 1% to 10% a dose-dependent drop in electrical resistance paralleled by an increase in permeability was observed. Ethanol produced a disruption of the tight junction protein ZO-1 as well as disassembly of cytoskeletal proteins such as actin and myosin. These changes proved reversible upon ethanol withdrawal. However, ethanol concentrations of 1% or above are only encountered in the duodenum/jejunum where concentrations of up to 5% have been reported[49], while ethanol concentrations in the ileum and colon tend to be much lower (0.2%-0.25%). This would entail that most of translocation of bacteria or bacterial products occurs in the upper gastrointestinal tract.

As mentioned above, human colonic bacteria have the capacity to metabolise alcohol to acetaldehyde[33,50] *via* bacterial alcohol dehydrogenase. Accordingly, colonic acetaldehyde concentrations in the millimolar range have been observed in rats[51] and piglets[52]. Acetaldehyde concentrations of 0.1-0.6 mmol/L led to a disruption of tight junctions and adherens junction *via* tyrosine phosphorylation of their main components[53].

In summary, there is substantial evidence that alcohol increases gut permeability to large molecules of the size of endotoxin and these effects may be due to a direct toxic effect on the mucosa of the proximal gut as well as molecular modifications at the level of interendothelial junctions. Likewise, acetaldehyde, as a result of alcohol metabolism by colonic bacteria, has the capacity to disrupt epithelial junctions, suggesting that the increased serum endotoxin concentrations observed in alcoholics may also be of colonic origin.

BACTERIA AND LPS IN PANCREATITIS

In the Western society, alcohol represents 70%-80% of cases of chronic pancreatitis. As stated earlier, experimental evidence suggests that bacterial endotoxin is an initiating factor for alcoholic pancreatitis[11,12]. In addition, bacterial translocation or the passage of bacterial products such as endotoxin into the systemic circulation appears to play a primary role in systemic spread, including multiple organ system failure and prognosis of the disease[54]. While endotoxin may be a key player at both ends of the disease spectrum, *i.e.*, as an initiating and aggravating factor of pancreatitis, the mechanisms leading to its increased presence in the blood may not be the same. In this chapter, both situations will be considered separately. The question as to whether bacteria or bacterial products (LPS) translocate will be addressed first.

Sepsis, a consequence of infected pancreatic necrosis, accounts for up to 80% of deaths in severe acute pancreatitis[55]. The germs most commonly cultured from infected pancreatic necrosis are gram negative bacilli presumably as a result of increased gut permeability[55,56]. Infection of pancreatic necrosis appears to be an early event occurring within a week after initiation of the disease in more than a quarter of patients undergoing necrosectomy[55,57]. However, the translocation of entire bacteria from the gut to the systemic circulation has not been proven so far in a setting of human acute pancreatitis. Indeed, blood cultures from patients with severe acute pancreatitis are often sterile even with established infected pancreatic necrosis[58]. Ammori *et al*[54] investigated the presence of bacterial DNA in the systemic circulation of 26 patients with acute pancreatitis. No bacterial DNA was detected in any of the samples. In one patient blood cultures subsequently turned out to be positive for *E. Coli*. This study suggests that translocation of entire bacteria, as opposed to bacterial products, rarely occurs

in acute pancreatitis. However, it has to be noted that the administration of prophylactic antibiotics to 9 of 19 patients with mild attacks and all 7 patients with severe attacks of pancreatitis may have prevented significant bacterial translocation.

Endotoxin is detectable in the majority of patients with established severe acute pancreatitis, in particular in more than 90% of patients dying of the disease[59,60]. Measuring circulating anti-endotoxin antibodies Barclay *et al*[61] have observed a significant decrease in antibody titres in patients with severe acute pancreatitis compared to patients with mild disease, suggesting higher endotoxin exposure in the former. In a comprehensive study, Ammori *et al*[8] undertook to measure intestinal barrier function (by measuring intestinal permeability using a PEG probe of 3350 Da) early in the course of acute pancreatitis and to examine the correlation between intestinal permeability, endotoxinaemia and disease severity. Intestinal permeability was significantly increased in patients with severe acute pancreatitis in comparison to mild disease and disease-free controls. Changes in permeability occured early in the course of the disease, before the development of multiple organ system failure. Endotoxinaemia correlated with intestinal permeability and was present more frequently and at higher concentrations in patients with severe disease. Similar observations were made by Windsor *et al*[9] demonstrating that a significant fall in serum concentrations of immunoglubulin G antiendotoxin core antibodies as a surrogate marker for endotoxemia in patients with acute pancreatitis was predictive of pancreatitis severity and multiple organ system failure.

LPS has also been reported to be a disease modifier in experimental non-alcoholic pancreatitis induced by various treatments. In a rat model of acute pancreatitis induced by the closed duodenal loop procedure[62] disease severity was significantly worsened by endotoxin administration[62]. Pastor *et al*[63] studied the direct effect of bacterial endotoxin on the course of caerulein-induced acute pancreatitis and pancreatitis-associated lung injury in TLR4 knockout mice and TLR4 sufficient controls. Administration of LPS alone did not induce pancreatitis per se nor did it potentiate the effects of cerulein on the pancreas in either mouse strain. However, there was a significant deterioration of pancreatitis-associated lung injury when LPS was combined with cerulein in wild type mice; lung injury was significantly reduced in TLR4 knockout mice implying that the effect of LPS was mediated *via* the TLR4 pathway[63]. Surprisingly, targeted deletion of TLR4 and CD14 in mouse models of cerulein- and Arginine-induced pancreatitis without LPS administration, resulted in attenuated pancreatitis and pancreatitis-associated lung injury[64]. The latter study suggests that "endogenous" endotoxin might play a role in the pathophysiology of these models or that LPS receptors play additional roles other than LPS signal transduction in pancreatitis.

The question whether endotoxinemia is an initiating event of *alcoholic* pancreatitis, similar to alcoholic liver disease has been approached in animal models. As noted earlier, it is well known that only a minority of alcoholics will ever develop acute pancreatitis suggesting that additional factors are required to elicit overt disease. This is evidenced by experimental work in rodents where long-term administration of ethanol did not lead to pancreatitis[65]. Fortunato *et al*[11] studied the effect of intravenous LPS administration on rats fed a Lieber-de Carli liquid diet with or without alcohol. Using single LPS doses of up to 3 mg/kg body weight, the authors showed a dose-dependent increase in pancreatic lesions, while rats fed alcohol alone did not display significant pancreatic damage. In accordance with the hypothesis whereby repeated attacks of acute pancreatitis lead to chronic disease (necrosis-fibrosis sequence proposed by Ammann *et al*[66]), Vonlaufen *et al*[12] showed that repeated weekly injections of endotoxin to alcohol-fed rats led to significant pancreatic fibrosis *via* a TLR4 mediated effect on pancreatic stellate cells (PSCs), the main effectors of pancreatic fibrosis. Moreover, the presence of TLR4 and its co-receptor CD14 was detected on disease-associated and normal human pancreatic stellate cells[12,67], suggesting that PSCs are a relevant target for endotoxin in human alcoholic pancreatitis.

Taken together, endotoxin (from gut derived bacteria) appears to be an aggravating factor of pancreatitis and associated extra-pancreatic organ damage regardless of aetiology. Furthermore, there is increasing (experimental) evidence that it may play a specific role in the initiation and progression of alcoholic pancreatitis.

THE GUT-LIVER-PANCREAS AXIS

In healthy humans, trace amounts of endotoxin may transiently enter the portal circulation and are cleared by Kupffer cells in the liver. When alcohol is consumed, the detoxifying capacity of the liver seems overwhelmed, since endotoxin is detected in the systemic circulation. In 1987, Bode *et al*[3] showed for the first time that gut-derived endotoxin is increased in the systemic circulation after acute alcohol consumption by subjects with or without liver damage. The authors evaluated peripheral venous blood endotoxin concentrations in patients with alcoholic and non-alcoholic cirrhosis and in a group of alcoholics with no evidence of chronic liver disease. Increased endotoxin concentrations were found in a significantly larger proportion of patients with alcoholic liver disease (67.3%) than patients with liver disease of non-alcoholic aetiology (45.5%, $P < 0.025$). Moreover, almost half of all subjects without preexisting liver disease, presenting after a single alcoholic binge, were found to have endotoxin in the blood; importantly, in this group endotoxinemia appeared to be a transient phenomenon with no endotoxin detected after 5-8 d. Further work by the same group confirmed elevated blood endotoxin levels in a significantly higher proportion of patients with alcoholic cirrhosis compared to patients with cirrhosis of a different cause. It is noteworthy, that mean blood endotoxin concentrations were significantly higher in cirrhotics of alcoholic aetiology (19 ± 2.3 *vs* 12 ± 3.1 pg/mL, *P*

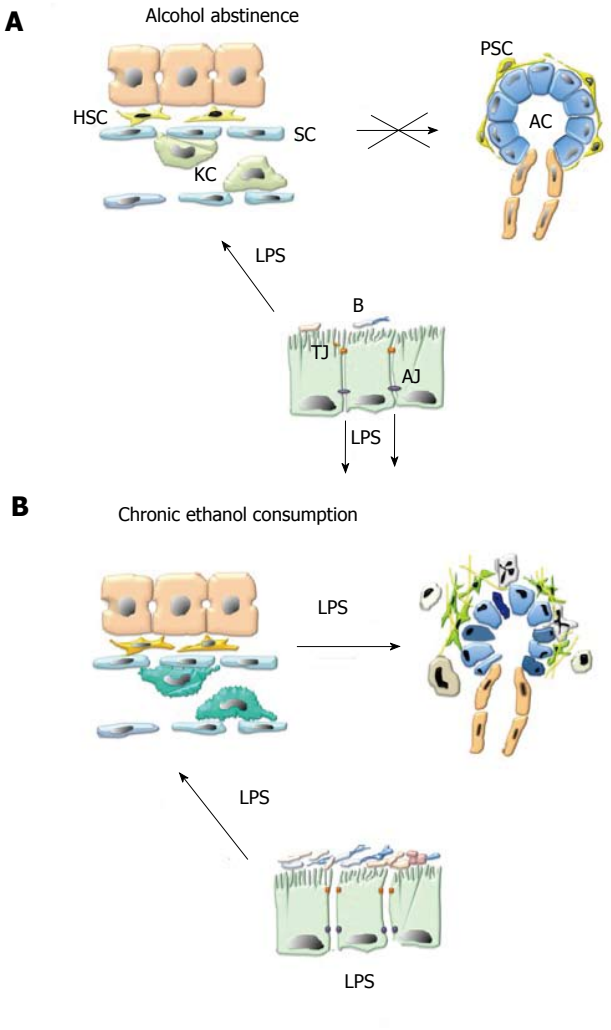

Figure 1 Alcohol and lipopolysaccharide promote pancreatic necroinflammation and fibrosis via pancreatic stellate cell activation. A: Alcohol abstinence. In healthy, non-alcoholic subjects small amounts of lipopolysaccharide (LPS) derived from the membrane of commensal gram negative bacteria (B) cross the gut epithelial barrier at the level of interendothelial junctions. LPS reaches the liver via the portal circulation where it is entirely cleared by Kupffer cells (KC) in the liver sinusoids (S), preventing it from entering the systemic circulation and reaching systemic organs such as the pancreas; B: Chronic ethanol consumption. Chronic alcohol consumption promotes bacterial proliferation in the proximal small bowel, dissociation of interendothelial junctions (by direct toxicity of alcohol and its metabolites) and leads to increased translocation of LPS into the portal circulation. In the liver, alcohol decreases the phagocytic capacity of Kupffer cells. As a result, LPS enters the systemic circulation and exerts its harmful effects on the pancreas. Alcohol and LPS promote pancreatic necroinflammation and fibrosis *via* PSC activation. TJ: Tight junctions; AJ: Adherens junctions; AC: Acinar cell; PSC: Pancreatic stellate cell.

< 0.025)[4].

Early work in patients with cirrhosis has reported toxic effects of alcohol on the reticulo-endothelial system, notably reduced phagocytic and metabolic activity of macrophages[68]. Experimentally, Kupffer cells from alcohol-fed rodents treated *in vitro* with ethanol at concentrations ranging from 10 to 100 mmol/L (corresponding to alcohol concentrations found in moderate drinkers and severe alcoholics respectively) displayed reduced endotoxin uptake and decreased production of the proinflammatory cytokine tumor necrosis factor alpha (TNF-α), an effect that was dose-dependent[69]. Endotoxin alone activates Kupffer cells by increasing their phagocytic capacity and inducing the production of proinflammatory cytokines (such as TNF-α and interleukin-6)[70].

Whether concomitant liver disease is a co-factor for alcoholic pancreatitis remains elusive. It is well known that patients with cirrhosis are predisposed to episodes of bacterial infections, including spontaneous bacterial peritonitis with bacteria of gut origin[71,72]. Liver disease impacts on small bowel motility (and potentially bacterial overgrowth), and this effect worsens with increasing severity of liver disease[73]. Experimentally, CCl_4-induced cirrhosis resulted in enterocyte oxidative stress, altered enterocyte mitochondrial function, increased lipid peroxidation and altered intestinal transport[74]. Part of the oxidative stress occurring in the enterocyte appears to be related to increased xanthine oxidase activity and increased intestinal permeability, a mechanism that can be blocked experimentally by the administration of xanthine oxidase inhibitors[75]. Accordingly, administration of allopurinol to patients with established cirrhosis efficiently reduced (systemic) oxidant stress, but did not have a significant effect on intestinal permeability[76].

Do alcoholic liver and pancreas disease occur together? A recent study by Yang *et al*[77] reviewing the epidemiology of alcohol-related pancreatic and liver disease in the United States, has reported that the prevalence of patients discharged with a diagnosis of both acute alcoholic pancreatitis and acute alcoholic hepatitis or both chronic alcoholic pancreatitis and chronic alcoholic liver disease was significantly lower than the prevalence of either disease alone. This is in conflict with necropsy data suggesting that subclinical damage to both organs often coexists[1].

PROPHYLAXIS AND SUPPORTIVE TREATMENT

Alcohol abstinence is the most obvious prophylaxis for alcoholic pancreatitis. Studies suggest that it reduces the incidence of acute attacks and retards clinical progression of the disease[78]. However, this goal is seldom reached and recurrence is common[79] (Figure 1).

Since bacteria or bacterial products appear to play a primary role in the initiation, progression and rate of complications of alcoholic pancreatitis, it appears logical to target gut bacteria either within the lumen *via* bacterial decontamination with nonabsorbable antibiotics or once translocation has occurred, *via* systemic administration of antibiotics.

Experimental evidence in rodents suggests that selective bacterial decontamination by oral, non absorbable antibiotics significantly reduced the incidence of pancreatic infection[80-82]. However, the application of prophylactic antibiotics in patients with acute pancreatitis has proven ineffective in a large randomized trial comparing the administration of meropenem *vs* placebo[83]. Another way to influence bacterial luminal content and act on gut

barrier integrity may be the application of probiotics (mostly lactobacilli or bifidobacterium strains), that is bacteria which exert protective effects on gut epithelial integrity and prevent colonization by pathogens[84]. However, in a large multicentre randomized controlled trial administration of a cocktail of probiotic bacterial strains (4 lactobacilli and 2 bifidobacteria)[85] within 72 h after onset of symptoms of pancreatitis was of no proven benefit. Moreover, excess mortality in the probiotic group was observed, with one third of deaths related to bowel ischemia. All of these patients presented with early organ failure. In a substudy it became apparent that administration of these particular probiotic bacterial strains in patients with multiple organ failure resulted in increased gut mucosal damage and permeability, as assessed by urinary intestinal fatty acid binding protein IFABP and NOx concentrations, while bacterial translocation was reduced in patients without organ failure[86].

Several animal and human studies have shown that enteral nutrition has a beneficial effect on gut mucosal integrity. In a recent meta-analysis by Petrov *et al*[87] including 5 randomised controlled trials in patients with severe acute pancreatitis, it was concluded that enteral feeding led to a significant reduction of pancreatic infections, other infectious complications and mortality, but not of organ failure. Another meta-analysis including 8 randomised controlled trials reached similar conclusions but also recorded a significant reduction in organ failure and need for surgical interventions in the total enteral nutrition (TEN) groups as compared to patients receiving total parenteral nutrition[87]. Despite overwhelming evidence in favour of early TEN in a setting of acute pancreatitis, the dogma that the diseased pancreas needs to be "put at rest" still prevails in many centers.

Taken together, early enteral nutrition significantly reduces infectious complications and mortality in patients suffering from acute pancreatitis regardless of aetiology. In contrast, the systematic administration of systemic antibiotics or of probiotics can not be recommended. To date, prophylactic studies aiming at inhibiting gut barrier dysfunction/bacterial translocation in alcoholic subjects are lacking.

CONCLUSION

There is now clear clinical and experimental evidence that bacteria and bacterial products such as endotoxin are associated with complications of pancreatitis. Furthermore, results of animal studies support the concept that bacterial endotoxin is an important factor in the initiation and progression of alcoholic pancreatitis.

Since all alcoholics may be expected to have bacterial translocation, the fact that only a minority develops overt pancreatitis indicates that genetic polymorphism plays a primordial role. Nonetheless, only two candidate genes (carboxylester lipase[88] and chymotrypsin C[89])-explaining a minoritiy of cases of alcoholic pancreatitis-have been identified so far. Additional case-control studies, comparing alcoholics with pancreatitis to alcoholics without pancreatic disease, and targeting genes encoding tight junctional proteins or LPS-receptors are needed to clarify the issue. Moreover, particular attention should be paid to the assessment of the quality of the microbiome in these two populations.

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TOPIC HIGHLIGHT

WJGP 5th Anniversary Special Issues (4): Barrett's esophagus

Low grade dysplasia in Barrett's esophagus: Should we worry?

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Abstract

The optimal management for low-grade dysplasia (LGD) in Barrett's esophagus is unclear. In this article the importance of LGD is discussed, including the significant risk of progression to esophageal adenocarcinoma. Endoscopic surveillance is a management option but is plagued by sampling error and issues of suboptimal endoscopy. Furthermore endoscopic surveillance has not been demonstrated to be cost-effective or to reduce cancer mortality. The emergence of endoluminal therapy over the past decade has resulted in a paradigm shift in the management of LGD. Ablative therapy, including radiofrequency ablation, has demonstrated promising results in the management of LGD with regards to safety, cost-effectiveness, durability and reduction in cancer risk. It is, however, vital that a shared-decision making process occurs between the physician and the patient as to the preferred management of LGD. As such the management of LGD should be "individualised."

Key words: Low grade dysplasia; Barrett's esophagus; Endoluminal therapy; Radiofrequency ablation; Esophageal adenocarcinoma

Core tip: Low-grade dysplasia (LGD) in Barrett's esophagus (BE) is an important entity and poses a significant risk of progression to esophageal adenocarcinoma. With the emergence of endoluminal therapy over the past decade there has been a paradigm shift in the management of LGD. Ablative therapy, such as radiofrequency ablation, has demonstrated promising results in the management of LGD with regards to safety, cost-effectiveness, durability and reduction in cancer risk. It is, however, critical that management should be through a shared-decision making process and "individualised". It is our belief that physicians should "*worry*" about LGD in BE.

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INTRODUCTION

Barrett's esophagus (BE) is an acquired condition, which represents an adaptive change to chronic gastro-esophageal reflux disease[1]. It is characterised by the presence of columnar mucosa within the tubular esophagus, which demonstrates specialized intestinal metaplasia (goblet cells). This metaplastic change is thought to represent a precursor for esophageal adenocarcinoma (EAC)[2]. It is postulated that there is a multi-step process during which the mucosa progresses through a metaplasia-dysplasia-carcinoma sequence[3]. Current guidelines, therefore, recommend endoscopic surveillance for patients with BE to detect early changes in the esophageal mucosa[4,5].

Dysplastic changes within the esophageal mucosa

include low-grade dysplasia (LGD) and high-grade dysplasia (HGD), which are regarded as intraepithelial neoplasia. Due to the high risk of progression to EAC[6] and the risk of coexisting EAC[7,8], the management of HGD includes either endoluminal therapy or an esophagectomy. Controversy, however, exists as to the optimal management for patients with LGD. In this article we discuss the evidence on the management of LGD and explain why we should "*worry*" about LGD.

LOW-GRADE DYSPLASIA: DEFINITION AND DIAGNOSIS

Dysplasia is defined as neoplastic epithelium that is confined within the basement membrane of the gland from which it arises differentiating it from invasive adenocarcinoma[9,10]. The revised Vienna classification standardizes the diagnosis of gastrointestinal epithelial neoplasia and adopts a five-tiered system when evaluating BE[11]. LGD is characterized by the relative preservation of glandular architecture but with cellular atypia (adenomatous or non-adenomatous changes) including nuclear hyperchromatism, pleomorphism, mucin depletion and absence of goblet cells. Identifying loss of surface maturation is important to aid in the differentiation between true dysplasia and regenerative atypia. In the presence, however, of inflammation/ulceration the epithelium may mimic that of LGD[12]. An important feature is the presence of crypt cells, which are significantly higher in number in patients with LGD who progress to EAC[13].

The Vienna classification system is reproducible amongst gastrointestinal pathologists and provides high specificity and predictive value even with LGD[14]. Even so the diagnosis of LGD can be difficult especially amongst non-gastrointestinal pathologists[15] especially when trying to differentiate between indefinite for dysplasia and LGD. Indeed the absence of well-defined cut off points with dysplasia makes such a differentiation difficult. Furthermore differentiating between LGD and HGD can also pose a diagnostic challenge with κ values for intra-observer and inter-observer variability being 0.64 and 0.45 respectively[16]. It is therefore recommended that pathologists who are experts in esophageal histopathology confirm the diagnosis of dysplasia in BE[4,5]. Consensus diagnosis of LGD among gastrointestinal pathologists[16] is vital as the degree of dysplasia is a key determinant for further management of patients with BE.

LGD AND PROGRESSION TO ESOPHAGEAL ADENOCARCINOMA

It is well established that the presence of dysplasia is associated with an increased risk of adenocarcinoma and in clinical practice it is the only recognised predictor of developing cancer. The neoplastic potential of LGD, however, is poorly defined. The development of cancer is associated with interplay of complex cellular, genetic and molecular mechanisms[3]. The natural history of dysplastic changes, therefore, is difficult to predict particularly on an individualised patient basis. This unpredictability serves further fuel to the argument that the diagnosis of dysplasia of any grade should be cause for concern.

Table 1 Molecular biomarkers predicting progression of dysplastic Barrett's esophagus

Molecular biomarker	Technique	Ref.
Overexpression of p53	IHC	[24-27]
Loss of heterozygosity (17p)	PCR	[28-30]
Hypermethylation of genes	PCR	[32]
Aneuploidy (2N)/Tetraploidy (4N)	Flow cytometry	[33-35]
Ki-67[1]	IHC	[23]

[1]Facilitates differentiation between non-dysplastic and dysplastic mucosa. IHC: Immunohistochemistry; PCR: Polymerase chain reaction.

It is largely assumed that a stepwise progression occurs from LGD to HGD and subsequent EAC, a sequence of events that was first proposed by Naef *et al*[17]. In clinical practice the timescale of this sequence is unknown and hence it may not be seen to occur; as such dysplastic BE of any grade could therefore progress to EAC. Evidence suggests that patients with LGD progress to EAC at a higher rate than patients with non-dysplastic BE. Two large population-based studies have demonstrated that the risk of progression for LGD is 0.5%-1.4%/year, in comparison to only 0.12%/year for non-dysplastic BE[18,19]. A large multicenter cohort study demonstrated that LGD persisted in 21% and progressed to HGD/EAC in 13%[20]. Although a significant number (66%) regressed, one may argue that a number of these may represent overdiagnosis or misdiagnosis rather than true regression. A more recent study demonstrated that the cumulative risk of progression to HGD or EAC was 85%, with an incidence rate of 13.4% per patient year for patients with confirmed LGD[21]. Whilst this statistic is alarming, it should be qualified by the observation by Curvers *et al*[21] that 85% of patients were downstaged from LGD to non-dysplastic BE. Thus discordance and limitations in pathological assessment make it difficult for physicians to make management plans based on histopathology alone. However, it has been demonstrated that when gastrointestinal pathologists make a consensus diagnosis of LGD the risk of progression to HGD or EAC is significant[16,22].

Due to the limitations of histological analysis, investigators have attempted to identify tissue biomarkers to help predict the risk of progression to EAC (Table 1). The cell cycle is dysregulated in dysplastic BE with abnormal expression of Ki67 on the surface epithelium, which aids in the differentiation of non-dysplastic and dysplastic BE[23]. It is, however, the overexpression of p53 in LGD that is associated with an increased risk of progression to HGD/EAC[24-26]. The concomitant diagnosis of aberrant p53 increased the positive predictive value of neoplastic progression from 15% to 33%[27]. Further the presence of 17p loss of heterozygosity (LOH), which is thought to represent inactivation of

p53 has been demonstrated to be a strong predictor of progression in BE[28]. Indeed LOH at the sites of known tumour suppressor genes (*APC*, *DCC*, *AND*, *TP53*) may be potential biomarkers of progression in BE[29,30]. As well as loci abnormalities, epigenetic changes including hypermethylation-induced inactivation of p16 have been demonstrated to be prevalent in BE[31] and associated with an increased risk of progression in LGD[32]. Hypermethylation of *RUNX3* and *HPP1* genes in BE may also represent risk factors for progression[32]. Flow cytometric analysis can also demonstrate DNA content abnormalities in patients with BE. The presence of aneuploidy or tetraploidy in patients with LGD is associated with an increased cumulative incidence of EAC[33-35]. There are, however, a number of caveats to the use of biomarkers in BE. Biomarker analysis is not universally applicable or feasible, especially in clinical practice. The current studies are potentially underpowered and there will undoubtedly be concerns regarding reproducibility between laboratories. There are also issues regarding costs and the requirement for complex analytical techniques including immunohistochemistry and flow cytometry. Indeed, the American Gastroenterological Association currently do not recommend the use of biomarkers to risk stratify patients with BE[5]. Nevertheless the above abnormalities in BE demonstrate promise in biomarker-based prediction and may reduce the inter-observer variability amongst pathologists. Further studies are necessitated before biomarkers can be utilised routinely in prediction of progression.

As well as biomarkers, the risk of progression is also related to clinical and endoscopic factors, including age, male gender, multifocality and length of the BE segment[18,36]. As LGD maintains a constant risk of progression to EAC[19] diagnosis at an early age is clinically relevant, as these individuals would have more life-years to potentially progress.

What is important, however, is the persistence of LGD with surveillance alone. Persistent LGD, a "pre-malignant lesion", only serves to further concern both the physician and patient and it is well established that BE has a significant decrement in health-related quality of life[37]. Anecdotally it is known that the natural history of dysplasia differs from patient to patient and this only adds to the inability to inform patients of their specific risk of neoplastic progression. If physicians are unable to accurately identify which patients with LGD will go on to develop HGD or EAC, surely intervention should be an option that is considered? Although most deaths are not cancer-related, a significant number of patients with LGD develop esophageal cancer[38], which in itself is associated with significant morbidity and burden to both the patient and the healthcare system.

LGD: SURVEILLANCE ALONE?

Guidelines currently recommend that patients with LGD undergo endoscopic surveillance every 6-12 mo until two consecutive biopsies demonstrate non-dysplastic BE[4,5]. Surveillance alone, however, is not without limitations. Firstly, and most importantly there has been no randomised, prospective trial demonstrating that surveillance has a survival advantage over no surveillance or intervention. The United Kingdom BOSS trial (DOI 10.1186/ISRCTN54190466) aims to answer this to a degree by establishing whether surveillance in BE (including LGD) is beneficial. In the meantime surveillance is based solely on a weak recommendation with moderate quality evidence[5].

For surveillance to have any survival advantage strict adherence to an endoscopic biopsy protocol (Seattle Protocol) is necessitated[39]. Adherence to such protocols has been demonstrated to be suboptimal, decreasing further with increasing length of BE and resulting in reduced detection of dysplasia[40,41]. Sampling error[42] and a mosaic of dysplastic and non-dysplastic areas are other key issues to be aware of. Standard high-resolution white light endoscopy only allows the detection of macroscopically obvious abnormalities. The adoption of narrow band imaging[43,44], autofluorescence imaging[44] chromoendoscopy and virtual chromoendoscopy[45,46] could significantly improve the detection of dysplasia. A promising technique is that of confocal laser endomicroscopy (CLE), which allows *in vivo* visualisation of the mucosal histology. CLE affords targeted biopsies, improving diagnostic yield even in the absence of macroscopic abnormalities[47,48]. Although CLE can improve the sensitivity of detecting mucosal changes, the technique is limited to tertiary-referral centres thus limiting its use in surveillance[49]. These advanced techniques need further validation, including a cost-benefit analysis before they can be routinely recommended for endoscopic surveillance.

Although not demonstrated HGD may co-exist amongst LGD and as such managing LGD with surveillance alone may be detrimental in such cases. More troublingly is that patients can develop HGD/EAC even with two consecutive biopsies revealing non-dysplastic BE[20]. Critically there is no prospective data to demonstrate that surveillance in BE is cost effective or improves mortality from EAC. All in all, strategies based on surveillance alone in LGD are exposed to limitations that can have far reaching implications. Further, patients' perceptions and concerns are important issues to consider with surveillance, especially with a premalignant condition. Crucially, following intervention for dysplasia, quality of life is improved through the perception that the risk of EAC is reduced[50].

As an adjunct to surveillance, chemopreventive strategies have been used in BE. The cornerstone of medical therapy is the proton-pump inhibitor (PPI), which is associated with a lower incidence of EAC[51] and is superior to H_2-receptor antagonists in reducing progression to dysplasia or EAC[52,53]. Interestingly, PPI therapy reduces cell proliferation in BE[54,55]. Evidence regarding PPI therapy is, however, indirect at best and merely associative. There is also a paucity of prospective, controlled clinical studies examining the role of PPI therapy in

BE and the development of EAC. Furthermore, even with symptom control persistent acid and bile refluxate is present in patients taking PPI therapy[56,57], thereby not eliminating the key factor in the pathogenesis of BE. Non-steroidal anti-inflammatory drugs and aspirin, which exert their effect by inhibition of the COX-1 and -2 enzymes may play a role in reducing progression to EAC[58,59]. In contrast selective inhibition of COX-2 (associated with colonic carcinogenesis) did not prevent progression of dysplasia to EAC[60]. It is clear that carcinogenesis in BE is a complex interplay of numerous factors, which may not necessarily be influenced by chemopreventive strategies. The results of the United Kingdom AspECT trial (ClinicalTrials.gov NCT00357682) are awaited and may help answer what role aspirin and PPI play in the progression of BE to EAC. Until then the American Gastroenterological Association do not recommend aspirin in patients with BE in the absence of cardiovascular disease.

LGD: ROLE OF ENDOLUMINAL THERAPY

The aim of endoluminal therapy is to eradicate both dysplastic BE and non-dysplastic BE, achieving reversion to neosquamous epithelium and thus reducing the risk of progression to EAC. Endoluminal therapies include endoscopic mucosal resection (EMR) for visible abnormalities (nodular BE) or ablative techniques such as radiofrequency ablation (RFA), photodynamic therapy (PDT) and argon plasma coagulation (APC).

It is currently recommended that EMR is an alternative to esophagectomy for patients with either HGD or intramucosal adenocarcinoma[5,61]. Further, EMR is also invaluable as both a diagnostic and staging procedure, the latter helping to differentiate between a mucosal or submucosal adenocarcinoma. Importantly, EMR significantly improves interobserver agreement on the diagnosis of both LGD and HGD in comparison to a standard biopsy technique[62]. However, there are no recommendations for the use of EMR for the management of LGD, particularly in the absence of a visible/nodular abnormality.

An early trial using PDT for ablation LGD showed promising results with an efficacy of 92.9%[63]. Further trials from the United Kingdom demonstrated that PDT was similarly efficacious in eradicating LGD[64,65]. Likewise a study utilising APC to ablate LGD demonstrated complete eradication of dysplasia at one year[66]. When comparing the two ablative therapies, PDT achieved higher rates of LGD eradication[67]. There are, however, concerns about the side effect profile of PDT with high stricture rates and photosensitivity being reported[63,68,69]. Of greater concern with any ablative technique is the risk of subsquamous intestinal metaplasia, which can develop into a subsquamous adenocarcinoma[68,70].

The ablation of intestinal metaplasia (AIM) trials, which adopted the technique of circumferential RFA (cRFA, Halo© 360) and focal RFA (fRFA Halo© 90), were pivotal in the management of both dysplastic and non-dysplastic BE. Initial studies were based on the identification of dose-response, safety and efficacy of cRFA in non-dysplastic BE[71]. A pilot study of patients with LGD, demonstrated that a combination of cRFA and subsequent fRFA (stepwise regimen) had a 100% complete response for dysplasia at 2-year follow up[72].

It was, however, the AIM dysplasia trial, which provided the first real evidence that RFA had a role in the management of LGD[73]. This prospective, multicenter, sham-controlled trial demonstrated that RFA resulted in complete eradication of LGD in 90.5% in comparison to 22.7% in the control group at 12 mo ($P < 0.001$). Eradication of non-dysplastic BE was demonstrated in 81% of patients undergoing RFA compared to 4% in the sham-control group. At follow-up with as required fRFA complete eradication of LGD was attained in 98% and 100% at 2- and 3-years respectively[74]. Importantly, for patients with LGD undergoing RFA overall disease progression was 2.04%/patient/year, with a 0.51%/patient/year progression rate to EAC[74]. The annual progression rate in sham-control group was 16.3%. This evidence demonstrated for the first time that endoluminal therapy in the form of RFA for dysplastic BE was potentially anti-neoplastic. Indeed no disease progression-related morbidity or mortality was demonstrated in this study.

More recently prospective studies from the United Kingdom[75] and the Netherlands[76] have verified the efficacy of RFA in eradicating dysplastic BE. The United Kingdom National Halo RFA Registry demonstrated following EMR (for nodular lesions), serial RFA eradicated dysplasia in 81% of patients at 12 mo with 94% remaining clear of dysplasia at 19 mo. Similarly, the smaller study from the Netherlands demonstrated following serial RFA (with or without EMR), 90% of patients remain in remission at 5-years.

There have, however, been concerns about the durability, risk of subsquamous intestinal metaplasia, safety and cost of RFA for dysplastic BE. For patients with LGD achieving complete eradication of dysplasia, 90% remained free of dysplastic BE and > 75% remained free of non-dysplastic BE at 3-years without additional RFA therapy[74]. Anti-reflux surgery (ARS), which reduces refluxate into the lower esophagus, may improve the durability of RFA. Understandably the elimination of acid reflux, a known risk factor for BE, may have a beneficial effect on neoplastic progression. Studies have demonstrated that concomitant fundoplication is safe, effective at eradicating dysplasia and improves durability when compared to RFA and subsequent PPI therapy[77,78]. There is, however, no data supporting the role of ARS as an anti-neoplastic intervention. It is clear that further prospective data is clearly necessitated to address the long-term durability of RFA with or without ARS. Our current understanding of the oncogenic potential of the neosquamous epithelium is limited. Yet it has been demonstrated this epithelium has no persistent molecular abnormalities (Ki-67, p53) or "buried" metaplasia following RFA. This is in contrast to other ablative techniques such as PDT where genetic abnormalities can

persist[79]. Although, the actual occurrence of subsquamous intestinal metaplasia post RFA is low[76] and can also occur without ablative therapy[80]. Furthermore, the incidence of subsquamous intestinal metaplasia is lower following RFA (0.9%) compared to PDT (14.2%)[80]. In the AIM dysplasia trial no perforations or procedure related deaths occurred over the 3-years. There were, however, a very small number of adverse events thought to be related to the procedure, with 7.6% of patients developing a stricture that required dilatation[74]. Although the incidence of adverse events is higher than that with endoscopy alone, it does vary with the type of procedure[81]. Indeed RFA has a better safety profile than PDT, which is associated with high rates of photosensitivity and stricture formation[68]. Ablative therapy has been shown to be cost-effective for HGD in a United Kingdom-based analysis[82]. Critics, however, question the cost-effectiveness of ablative therapy for LGD in comparison to surveillance. In a cost-utility analysis, if ablative therapy could eradicate more than 28% of LGD, ablation would be favoured over surveillance[83]. Furthermore RFA is only cost-effective in patients with confirmed and stable LGD[84], which defines the importance of consensus agreement for LGD. Evidently the cost-effectiveness depends on the durability of ablative therapy. Discontinuation of surveillance would reduce long-term costs, but this is not recommended as recurrence (dysplastic and non-dysplastic) can occur[85,86] Thus following ablative therapy, surveillance is recommended in all patients to identify potential changes in the mucosa.

CONCLUSION

The emergence of endoluminal therapy over the past decade has resulted in a paradigm shift in the management of dysplastic BE. As such, the American Gastroenterological Association has recommended that RFA is a therapeutic option for patients with confirmed LGD[5].

Critics, however, claim that there are caveats to this recommendation. Firstly there are concerns regarding the diagnostic uncertainty with LGD, in particular the inter- and intra-observer variability amongst pathologists. As such, ablative therapy may result in over-treating patients who merely have non-dysplastic BE. The natural history of LGD is unclear and the literature demonstrates marked heterogeneity, especially with regards to progression risk. It is thought that patients with LGD and non-dysplastic BE have a similar low risk of developing EAC[20]. However, if patients with BE are truly being overdiagnosed, this would mean that studies looking at the natural history of LGD are being "contaminated" with non-dysplastic BE leading to an underestimation of progression and malignant potential. Thus, all patients diagnosed with LGD require a consensus from two or more gastrointestinal pathologists.

The purpose of any intervention for LGD is to reduce the incidence of EAC. Trials have demonstrated short-term benefits for ablative therapy, but critics claim that there is no long-term data demonstrating the prevention of EAC. Indeed there is paucity of long-term data but a recent meta-analysis demonstrated that ablative therapy reduced the risk of EAC in patients with LGD[87]. There is, however, heterogeneity amongst the literature and this reflects the molecular and biological differences in dysplasia amongst patients.

Finally opponents of ablative therapy for LGD, claim the side-effect profile does not justify intervention over surveillance alone. Furthermore, ongoing surveillance is necessitated following ablation and as such has an impact on the cost-effectiveness and quality of life. Although PDT has an unfavourable side-effect profile, RFA has been demonstrated to be safer and better tolerated. The requirement of ongoing surveillance will no doubt be addressed once the long-term efficacy and durability of RFA has been established. Results from an ongoing randomised trial (ClinicalTrials.gov NCT01360541) comparing RFA against surveillance for LGD will provide answers to the queries posed by opponents to ablative therapy

Despite the above caveats it is the authors' belief that consensus defined LGD is an important entity and warrants consideration of ablative therapy. The authors believe that management of LGD should be "individualised" and based on known risk factors for progression. Indeed the panacea would be to identify reliable biomarkers or predictors of progression to EAC. However, until then we need to rely on clinically relevant factors to help with risk stratification. Thus a young, male patient with long segment BE and multifocal LGD would be regarded as "high risk" and should therefore be considered for ablation. It is, however, not as simple as that in clinical practice and the uncertainty with progression should encourage physicians to consider ablative therapy as an alternative to surveillance alone. Most importantly as per the American Gastroenterological Association's recommendation there should be shared-decision making process between the physician and the patient as to the preferred management of LGD.

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REVIEW

Endoscopic surveillance strategy after endoscopic resection for early gastric cancer

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Abstract

Early detection of early gastric cancer (EGC) is important to improve the prognosis of patients with gastric cancer. Recent advances in endoscopic modalities and treatment devices, such as image-enhanced endoscopy and high-frequency generators, may make endoscopic treatment, such as endoscopic submucosal dissection, a therapeutic option for gastric intraepithelial neoplasia. Consequently, short-term outcomes of endoscopic resection (ER) for EGC have improved. Therefore, surveillance with endoscopy after ER for EGC is becoming more important, but how to perform endoscopic surveillance after ER has not been established, even though the follow-up strategy for more advanced gastric cancer has been outlined. Therefore, a surveillance strategy for patients with EGC after ER is needed.

Key words: Early gastric cancer; Endoscopic resection; Synchronous gastric cancer; Metachronous gastric cancer; Surveillance

Core tip: Recent advances in endoscopic modalities and treatment devices may make endoscopic treatment, such as endoscopic submucosal dissection, a therapeutic option for early gastric cancer (EGC). Consequently, short-term outcomes of endoscopic resection (ER) for EGC have improved. Therefore, surveillance with endoscopy after ER for EGC is becoming more important, but how to perform endoscopic surveillance after ER has not been established, even though the follow-up strategy for more advanced gastric cancer has been outlined. In this review, we discuss clinical problems in surveillance after ER for EGC.

Nishida T, Tsujii M, Kato M, Hayashi Y, Akasaka T, Iijima H, Takehara T. Endoscopic surveillance strategy after endoscopic resection for early gastric cancer. *World J Gastrointest Pathophysiol* 2014; 5(2): 100-106 Available from: URL: http://www.wjgnet.com/2150-5330/full/v5/i2/100.htm DOI: http://dx.doi.org/10.4291/wjgp.v5.i2.100

INTRODUCTION

Gastric cancer is the second most common cause of death from cancer worldwide[1,2], and more than half of the world's gastric cancer cases arise in Eastern Asia. Early gastric cancer (EGC) is typically small and asymptomatic and has a good prognosis[3,4], but advanced gastric cancer has a higher mortality rate[5]. Therefore, early detection and treatment could contribute to improved prognoses for patients with gastric cancer. Screening with endoscopy and biopsy sampling is important for patients with premalignant lesions and may lead to early cancer detection[6,7]. In Japan, a mass-screening program for gastric cancer is conducted on a nationwide scale because of the high prevalence of gastric cancer. Such a screening program may help to detect EGC that is treated by endo-

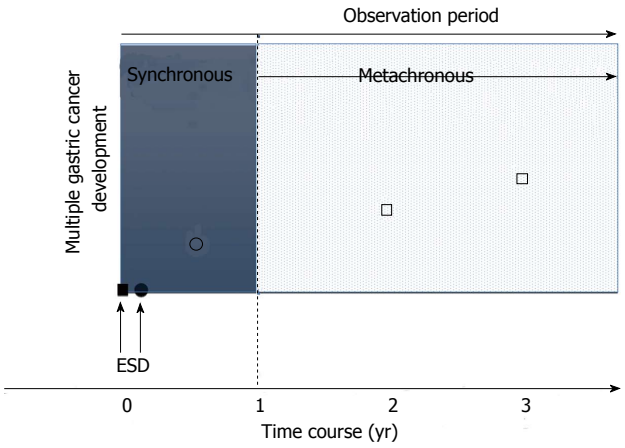

Figure 1 Definition of multiple gastric cancer development. Synchronous (within 1 year) or metachronous cancer (□) according to the time at which the multiple cancers developed. Synchronous cancer is also classified as "concomitant cancer" (●) or "missed cancer" (○). ■: Primary gastric cancer.

scopic resection (ER).

Japanese guidelines classify EGC into the following three groups, as proposed by Gotoda *et al*[8], when considering the indication of ER for EGC: the "guideline group", the "expanded guideline group" and the "non-curative group". Based on the tumor characteristics, the guideline group is defined as mucosal differentiated cancer with the largest diameter measuring < 20 mm. In Japan, ER is definitely indicated for this group. If the lesion meets Japanese guideline criteria and R0 resection is achieved, it is classified as a curative tumor, which does not require need further intense follow-up because it has a negligible risk for lymph node or distant metastasis[9-11]. Moreover, with the advancement of endoscopy and high-frequency generators, endoscopic submucosal dissection (ESD) has been developed. Consequently, the short-term outcomes of ER for EGC have improved[12,13].

However, patients who have undergone ER for EGC are considered at high risk for having other gastric cancer lesions. The incidence of local recurrence is decreasing because of ESD, which enables the evaluation of the horizontal and vertical margins of the resected specimen. Therefore, the risk of secondary gastric neoplasms developing during the follow-up period after ER has become a serious problem. In this review, we discuss clinical problems in developing a secondary gastric cancer after ER in patients with EGC, except for patients with non-curative resection based on Japanese gastric cancer treatment guidelines[14], with the goal of targeting synchronous and metachronous multiple gastric cancer development after ER.

GASTRIC CANCER RISK IN PATIENTS WITH *HELICOBACTER PYLORI* INFECTION

Stomach carcinogenesis is generally considered to originate from chronic active inflammation of the stomach mucosa caused by *Helicobacter pylori* (*H. pylori*) infection, followed in an ideal model by atrophy, intestinal metaplasia and dysplasia or adenoma, some of which eventually develop into gastric adenocarcinomas[15]. The incidence range of gastric adenocarcinoma in patients with atrophic gastritis or intestinal metaplasia is 0.1%-0.5%[7,16]. In particular, elderly persons often have multiple gastric cancers because individuals older than 65 have advanced degrees of intestinal metaplasia, a high risk for developing gastric cancer[17]. Yoshida *et al*[18] indicated that a high serum pepsinogen level and a high *H. pylori* antibody titer were risk factors for developing cancer in *H. pylori*-infected subjects from a large cohort of 4655 healthy subjects. The risk of developing gastric cancer cannot be abolished even if *H. pylori* is successfully eradicated[19]. However, the prevalence of gastric cancer in subjects who have not been infected with *H. pylori* is very low. Matsuo *et al*[20] calculated a gastric cancer prevalence of 0.66% (95%CI: 0.41-1.01) in the Japanese population without *H. pylori*.

DEFINITIONS OF SYNCHRONOUS AND METACHRONOUS MULTIPLE GASTRIC CANCER DEVELOPMENT

Even patients after curative ER for EGC have higher risks of multiple cancer development than patients with atrophic gastritis or intestinal metaplasia without past EGC. The doubling time of EGC is relatively long, ranging from 1.6 to 9.5 years[21]. Therefore, some occult lesions in the stomach might be observed when detecting a first EGC. Moreover, detecting secondary cancer after initial ER depends on how often the surveillance endoscopy is performed, which can include a lead-time bias. It is difficult to determine whether a secondary cancer is synchronous and metachronous gastric cancer. Until now, there have not been strict definitions of these lesions after ER.

In this review, we define multiple gastric cancer development as synchronous (within 1 year) or metachronous cancer according to the time at which the multiple cancers develop. Moreover, synchronous cancer is classified as "concomitant cancer" or "missed cancer". Concomitant cancer is defined as multiple cancers that had already been detected and diagnosed before the initial ESD. In recent reports, there is a consensus that cancers detected within 1 year after the initial ER should be regarded as 'missed' synchronous cancers[22,23]. We define missed cancer as cancer that is detected within 1 year, except for concomitant cancer (Figure 1).

CONCOMITANT AND MISSED SYNCHRONOUS GASTRIC CANCER AFTER ER

There are many reports about synchronous gastric cancer in surgically resected stomachs, with an incidence ranging from 4.8% to 14.6%[24-27] (Table 1). In addition,

Table 1 Incidence of synchronous gastric cancers in the surgically resected stomach

Ref.	Overall		Missed lesion	
Noguchi *et al*[42], 1985	6.50%	468/7220		
Ezaki *et al*[24], 1987	14.60%	75/512		
Honmyo *et al*[43], 1989	4.80%	40/839	53%	21/40
Mitsudomi *et al*[44], 1989	8.30%	83/997	23%	42/182
Kosaka *et al*[25], 1990	5.80%	49/852		
Kodera *et al*[26], 1995	5.70%	160/2790	53%	85/160
Kodama *et al*[45], 1996	6.80%	107/1458	64%	69/107
Fujita *et al*[46], 2009	8.70%	266/3042		
Lee *et al*[27], 2010	5.20%	51/986	28%	14/51
Total	6.90%	1299/18696	39%	210/540

Table 2 Incidence of synchronous gastric cancers in the endoscopically resected stomach within 1 yr of the initial endoscopic resection

Ref.	Overall		Missed lesion
Arima *et al*[23], 1999	6.60%	5/76	NA
Nasu *et al*[10], 2005	11%	16/143	NA
Nakajima *et al*[9], 2006	9.20%	58[1]/633	NA
Kobayashi *et al*[28], 2010	19.20%	45/234	NA
Han *et al*[29], 2011	4%	7/176	NA
Kato *et al*[19], 2013	8.70%	110/1258	19% (21/110)
Kim *et al*[47], 2013	2%	12[2]/602	NA
Total	8.10%	253/3122	

[1]Including 14 adenomas; [2]Including 5 adenomas. NA: Not available.

the incidence of synchronous multiple gastric cancers among the patients treated by ER ranges from 1.2% to 19.2%[10,19,28,29] (Table 2). In our large cohort, synchronous cancer was detected in 110 patients within 1 year after ESD [8.7% (110/1258 patients)]. Twenty-one out of 110 patients (19%) were considered to have missed cancers because these lesions were not detected at the preoperative endoscopic evaluation before initial ESD. The overall rate of missed cancer was 1.7% (21/1258)[19]. In surgically resected cases, missed synchronous cancer cases range from 23% to 64% of gastric cancers (Table 1). Compared with surgical cases, our missed rate was lower because it makes a difference whether a gastric cancer is in the early or advanced stage. Therefore, we should keep in mind that the missed rate was not negligible and that we need an endoscopic surveillance strategy that addresses the problem of missed cancer.

Four of 21 missed lesions (19%) were massively invading cancers (including one advanced cancer) in our study[19], which suggests that we should perform preoperative screening carefully and should consider missed cancer as a problem because we tend to focus on the initial lesion. To predict missed cancers, we found that the endoscopist's experience was an independent predictor of missed cancer. However, Lee *et al*[27] reported that expert endoscopists can miss other lesions in as many as 27.5% of patients and that smaller size was correlated with missed lesions. It might be difficult to decrease the number of missed lesions in the near future despite recent endoscopic advances, such as image-enhanced endoscopy and magnifying endoscopy. Therefore, we should pay special attention to the possibility of missed cancers, not only initially detected lesions at the first evaluation, and the first surveillance EGD should be performed soon after the ESD so as not to miss cancers.

Table 3 Metachronous cancer rate after endoscopic resection

Ref.	Rate		Follow up period (yr)
Arima *et al*[23], 1999	7.90%	6/76	7[1]
Nasu *et al*[10], 2005	14%	20/143	4.8 (median)
Nakajima *et al*[9], 2006	8.40%	53/633	4.4 (mean)
Kim *et al*[48], 2007	2.70%	13/479	3.3 (median)
Kobayashi *et al*[28], 2010	12.80%	30/234	5 (median)
Lee *et al*[49], 2011	3.30%	15/458	2.2 (median)
Kato *et al*[19], 2013	5.20%	65/1258	2.2 (mean)
Total	6.70%	202/3281	

[1]All patients were followed up for 7 yr.

METACHRONOUS GASTRIC CANCER AFTER ER

In reports conducted on patients with surgically resected stomachs in the remnant stomach after surgery for gastric cancer, the rate of metachronous gastric cancer ranges from 1.8% to 5%[30-32]. Therefore, the remnant stomach is at high risk for developing metachronous gastric cancer. ER contributes to preserving the stomach compared with surgically resected stomach and maximizing quality of life. Therefore, patients with EGC resected by ER are considered at higher risk for developing metachronous gastric cancer than surgically resected patients because the former have more remnant stomach and tend to survive longer. The metachronous cancer rate after ER ranges from 2.7% to 14% (Table 3). Nakajima *et al*[9] reported that metachronous gastric cancer had an overall incidence of 8.2% (52 out of 633 patients) and that the annual incidence was constant (cumulative 3-year incidence 5.9%). The average time to detect a first metachronous gastric tumor after the initial ER was 3.1 ± 1.7 years (range, 1-8.6 years)[9]. We also found that the cumulative incidence curve revealed a linear increase. The cumulative incidence rates of metachronous cancers at 2, 3, 4 and 5 years were 3.7%, 6.9%, 10% and 16%, respectively. Based on these data, the metachronous gastric cancer incidence curve, except for synchronous cancer, seems to increase linearly by 3%-3.5%[9,19,33].

LOCAL RECURRENCE AFTER ER

Conventional endoscopic mucosal resection (EMR) techniques are associated with the risk of local recurrence because it is difficult to achieve *en bloc* resection, in particular with larger lesions. Until recently, EMR was widely accepted as a useful, standard treatment for gastrointestinal tract neoplasms, but EMR has been replaced by ESD because *en bloc* resection of specimens larger than 20 mm is difficult to perform with EMR. Local recurrence

Table 4 Local recurrence rate after endoscopic resection

Ref.	Local recurrence rate			
	EMR		ESD	
	Curative	Not curative	Curative	Not curative
Oka *et al*[50], 2006	2.90%	4.40%	0%	0%
Kim *et al*[48], 2007[1]	6.0% (24/399)	15% (10/68)		
Park *et al*[11], 2010	18% (9/50, not *en bloc*; 18)		3.7% (7/189, not *en bloc*: 25)	
Lee *et al*[49], 2011	NA	NA	0.7% (2/276, not *en bloc*: 3)[2]	
	NA	NA	0% (0/182, not *en bloc*: 22)[3]	
Kato *et al*[19], 2013	NA	NA	0.4% (5/1258)	
Tanabe *et al*[51], 2013[4]	4.2%(15/359)[5]		0.2% (1/421)	

"Not curative" includes piecemeal resection or marginal positive resection. [1]Including 34 lesions treated by ESD (6.6%); [2]Guideline group; [3]Expanded guideline group; [4]For lesions meeting the JGCA criteria, the local recurrence rates were 2.9% in the EAM group and 0% in the ESD group; [5]Treated by endoscopic aspiration mucosectomy (EAM). EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection; NA: Not available.

strongly depends on whether the initial lesion is completely resected. With piecemeal resection or marginal-positive resection (not curative), local recurrence ranges from 4.4% to 18% (Table 4). Using ESD, *en bloc* marginal-negative resection can be performed with larger specimens. Developing local recurrence after complete *en bloc* resection in mucosal gastric cancers occurs rarely. In fact, our study revealed that local recurrence was seen in only 0.40% of patients (5/1258)[19]. This rate was quite low, but not zero. Park *et al*[11] also reported complete *en bloc* resection in one patient who developed local recurrence after complete resection by ESD. It is speculated that it is difficult to detect a very small concomitant lesion or precancerous lesion near the initial ESD site at initial evaluation or that detection depends on the status of the resected specimen reviewed by pathologists or each pathologist's experience. To evaluate resected specimens properly, the ER specimen should be cut parallel to the closest margin direction. When the negative margin is obvious, the specimens are step-sectioned along the minor axis of the specimen to obtain more information. The Japanese Gastric Cancer Association recommended that a section width of 2 mm allows for a more accurate diagnosis. We should remember that complete resection does not exclude the possibility of local recurrence in cases where R0 resection is achieved.

INTERVENTION FOR SECONDARY CANCER AFTER ER OF GASTRIC CANCER

In a study by our group, 169 of 175 secondary cancers (97%) after ESD were treated by re-ESD[19]. Among these cancers, 164 lesions were diagnosed as fitting the guideline or expanded guideline group and were followed up without additional treatment. Of the remaining five lesions, two were diagnosed as mucosal undifferentiated adenocarcinomas, and three were diagnosed as submucosal cancers after ESD; these patients then underwent additional gastrectomies. In addition, six lesions were treated by gastrectomy. Of these cases, four were pathologically diagnosed as belonging to the guideline or expanded guideline group after gastrectomy, and the remaining two were pathologically diagnosed as non-curative. Altogether, seven lesions were diagnosed as non-curative: three were intramucosal undifferentiated cancers, and four were massively invading cancers. Nakajima *et al*[9] concluded that frequent follow-up examinations negatively affect a patient's quality of life and result in an increase in overall medical expenses. Similarly, we also found that almost all secondary cancers after ESD were treatable by re-ESD[19]. Nakajima *et al*[9] reported that almost all first metachronous gastric cancers (96.2%) were treated curatively with re-ER. Considering those re-ER rates for metachronous cancer (96.2%, 97%), most metachronous secondary cancers can be non-surgically treated after the follow-up endoscopy.

HANDLING OF GASTRIC HIGH- AND LOW-GRADE INTRAEPITHELIAL NEOPLASMS

Gastric intraepithelial neoplasia, also called dysplasia or adenoma, is considered to be a precancerous lesion with a variable clinical course. The natural course of gastric intraepithelial neoplasia remains unclear. In particular, it is difficult to differentiate dysplasia/adenoma and adenocarcinoma using biopsy specimens because of the inaccuracy of obtaining a biopsy specimen from a malignant region of an adenoma[34,35]. Previous prospective long-term follow-up studies indicated that the gastric cancer-developing incidence in low-grade intraepithelial neoplasms (LGIN) is approximately 10%[35]. This low risk of malignant transformation compared to high-grade intraepithelial neoplasia (HGIN) may be due to the slowly progressive natural course of LGIN and supports a follow-up strategy. Once developing HGIN is diagnosed from biopsy specimens, 90% of them are ultimately diagnosed as adenocarcinoma after ER[36]. Generally, it is recommended that category 4 lesions (based on the Vienna classification: high-grade dysplasia and intramucosal cancer) be resected because they have a high potential for progression to adenocarcinoma[35]. Our current knowledge based on initial endoscopic intervention - not follow-up - indicates that over 40% of LGINs are diagnosed as adenocarcinoma after ER. Considering the high incidence of adenocarcinoma in HGIN, it could be recommended that ER be considered an indication for HGIN detected as a secondary lesion after ER. We are currently evaluating whether ESD is a valid strategy for gastric intraepithelial neoplasms with regard to safety and cost-effectiveness (UMIN Clinical Trials Registry: http://www.umiNAc.jp/ctr/, number UMIN000007476).

H. PYLORI ERADICATION

Extensive epidemiologic studies have shown that *H. pylori* infection is a major risk factor for developing gastric cancer[37]. According to most retrospective case-control and prospective epidemiologic studies, the risk of developing gastric cancer is two- to six-fold higher in patients with *H. pylori* infection than in patients without *H. pylori* infection[38]. Furthermore, some of the trials eradicating *H. pylori* have shown that successful eradication reduces the frequency of gastric cancer in high-risk populations, but *H. pylori* eradication may not completely abolish the risk for gastric carcinogenesis[39]. Therefore, *H. pylori* eradication might reduce secondary cancer after ER. Fukase *et al*[33] prospectively reported that prophylactic eradication of *H. pylori* after ER of EGC reduced secondary metachronous cancer by approximately one-third (OR = 0.353). Therefore, it is highly recommended that *H. pylori* be eradicated after ER for EGC. Based on Fukase's report, as of 2010, Japanese health insurance is allowed to cover *H. pylori* eradication therapy after ER for EGC. However, some retrospective cohort studies report no difference in the rate of metachronous cancer between patients who undergo successful *H. pylori* eradication and those who do not receive eradication treatment[19,40,41]. Therefore, because of the short 3-year observation of Fukase's report, whether *H. pylori* eradication reduces metachronous recurrence after ER for EGC is considered controversial. We speculate that the requirement for *H. pylori* eradication depends on how many high-risk patients have synchronous or metachronous recurrence. Therefore, it is important to conduct annual surveillance endoscopies after ER in patients with or without successful eradication, though patients with successful eradication will require longer surveillance until it is clear how long and how often surveillance endoscopy needs to be performed.

SURVEILLANCE STRATEGY FOR SECONDARY CANCER AFTER ER OF GASTRIC CANCER

There are no randomized trials to guide surveillance strategies after curative EGC resection. The 2013 consensus-based guidelines from the National Comprehensive Cancer Network (NCCN) suggest the same follow-up strategy that is used for more advanced disease, regardless of treatment type (NCCN Guideline version 2, 2013, http://www.nccn.org/professionals/physician_gls/f_guidelines.asp). The guidelines state that even for Tis or T1 with N0 lesions achieving R0, all patients should be followed up systematically, and follow-up should include a complete history and physical examination every 3 to 6 mo for 1 to 2 years, every 6 to 12 mo for 3 to 5 years and annually thereafter, along with other advanced stages. However, it is important to consider the curability of the initial ER. In Japan, ER is definitely indicated for guideline groups according to Japanese guideline criteria[14]. If the lesions meet the Japanese guideline criteria and R0 resection is achieved, the lesion is classified as a curative group and does not require further intense follow-up because it has a negligible risk for lymph node or distant metastasis[9-11].

Therefore, we recommend the following surveillance strategies: (1) an endoscopist who has performed at least 500 esophagogastroduodenoscopies should perform the preoperative screening; (2) intensive (every 6 mo) surveillance is preferred in the first year after ER to detect missed concomitant invasive cancers; and (3) annual surveillance should be performed for at least 5 years after the ER. From the viewpoint of avoiding gastrectomy and preserving most of the stomach and quality of life, it might not be important to strictly define the difference between synchronous and metachronous gastric cancer.

At this time, it is unclear whether the developing metachronous cancer is self-limiting or permanent. In report by Kobayashi *et al*[28], which included a follow-up longer than 10 years, showed that the metachronous recurrence curve reached a plateau and that the risk was not continuous after 10 years. In the future, the validity of our recommendations should be confirmed with a prospective study, and it is necessary to evaluate whether metachronous cancer is self-limiting.

CONCLUSION

It has not yet been established how endoscopic surveillance after curative ER should be performed. The rate of synchronous multiple gastric cancers among patients treated by ER is < 20%. After 1 year, the metachronous gastric cancer incidence increases linearly at an approximate rate of 3% per year. However, approximately 96% of patients with developing metachronous cancer were treated curatively with re-ER. Considered together with the population of ESD and advances in endoscopy, local recurrence or missed cancer may be negligible. Therefore, it might not be necessary to perform intensive endoscopy surveillance within 1 year to detect local recurrence. Surveillance endoscopies can permit the endoscopic treatment of cancers that may have been missed or that develop later.

In conclusion, skilled endoscopists should perform preoperative screening before initial ESD. We recommend that intensive (every 6 mo) surveillance be performed in the first year after ER to detect missed concomitant invasive cancers, and then annual surveillance should be performed for at least 5 years. In the future, it should be clarified whether longer surveillance is necessary.

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MINIREVIEWS

Role of gamma-delta T cells in liver inflammation and fibrosis

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Abstract

Conventional adaptive T cell responses contribute to liver inflammation and fibrogenesis, especially in chronic viral infections and autoimmune hepatitis. However, the role of unconventional gamma-delta (γδ) T cells in liver diseases is less clear. In the past two decades, accumulating evidence revealed that γδ T cell numbers remarkably increase in the liver upon various inflammatory conditions in mice and humans. More recent studies demonstrated that the functional effect of γδ T cells on liver disease progression depends on the subsets involved, which can be identified by the expression of distinct T cell receptor chains and of specific cytokines. Fascinatingly, γδ T cells may have protective as well as pathogenic functions in liver diseases. Interferon γ-producing γδ T cells, for example, induce apoptosis in hepatocytes but also in hepatic tumor cells; while interleukin-17-expressing γδ T cells can downregulate pathogenic effector functions of other immune cells and can promote apoptosis of fibrogenic stellate cells. However, the results obtained in human liver disease as well as murine models are not fully conclusive at present, and the effects of γδ T cells on the outcome of liver disease might vary dependent on etiology and stage of disease. Further definitions of the γδ T cell subsets involved in acute and chronic liver inflammation, as well as their effector cytokines might uncover whether interference with γδ T cells could be a useful target for the treatment of liver disease.

Key words: Liver fibrosis; Liver cirrhosis; Interleukin-17; Gamma/delta T cells; Cytokines

Core tip: The liver is particularly enriched in unconventional T cells expressing the gamma-delta T cell receptor and the functional role of these gamma-delta (γδ) T cells in liver diseases is being intensively investigated at present. γδ T cells accumulate in inflamed liver and their function appears highly dependent on the distinct subsets. In principle, γδ T cells can be protective as well as pathogenic in the context of liver inflammation. This review summarizes the current knowledge of γδ T cell effector functions and the cytokines produced by these cells in human liver diseases and murine experimental models of acute and chronic liver injury.

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INTRODUCTION

Despite its various metabolic functions, the liver is also an important immunological organ. The blood coming from the gastrointestinal tract *via* the portal vein carries manifold potential antigens, derived from the commensal microflora of the gut, food or invading pathogens[1]. Hepatic leukocytes are able to either mount immune responses against pathogenic antigens or to induce tolerance against harmless substances[2]. Innate immune cells are important

triggers of hepatic inflammation and it is well known that the liver is selectively enriched in macrophages (Kupffer cells), natural killer (NK) and natural killer T (NKT) cells, and also one of the richest sources for gamma/delta T cells (γδ T cells) in the body[3,4]. About 15%-25% of the hepatic T cells express the gamma/delta T cell receptor (TCR), indicating that this specific lymphocyte population might exert important functions in liver homeostasis and diseases. Moreover, the liver is also a site of extrathymic generation of γδ T cells during human fetal development, where the first transcripts of γδ TCR genes appear before a functional thymus is developed[5]. γδ T cells are a specific subpopulation of non-conventional T cells that are identified by expression of the γδ TCR instead of the αβ TCR[6,7]. In secondary lymphoid organs they account for only 2%-3% of all $CD3^+$ cells, while the highest abundance of γδ T cells is seen in the gut mucosa[8].

γδ T cells are often described to link innate and adaptive immunity as they share features with innate immune cells as well as with conventional T cells of the adaptive immune system[9,10]. In contrast to αβ T cells, γδ T cells leave the thymus after their maturation as mature T cells with a defined functional potential in a so-called preactivated status[11]. Although γδ T cells are able to recognize antigens presented on MHC molecules, they express only a restricted TCR repertoire and also recognize a lot of non-peptide ligands without the need for TCR engagement[12,13]. In the periphery, γδ T cells can also be sufficiently activated through cytokines without TCR engagement, allowing them to respond much faster than αβ T cells. Similar to conventional T cells, γδ T cells can kill target cells *via* death receptor mediated apoptosis or release of cytolytic granules[14,15]. They also produce large amounts of immunomodulatory cytokines, including interferon (IFN)γ, interleukin (IL)-17, IL-4, IL-5, IL-10, IL-13, TGFβ and GM-CSF[16].

According to their functional potential, γδ T cells can be subdivided into different effector populations. γδ T cells expressing a specific cytokine or with particular tissue localization often show a bias towards use of the same TCR V gene segments. IFNγ secreting γδ T cells, for example, often express Vδ1 or Vγ9Vδ2 chains[17-19], while γδ T cells expressing Vγ4 are frequently associated with production of IL-17[20,21] and/or IL-10[19]. In mice, these subtypes can also be distinguished by expression of surface markers, with the IFNγ secreting subpopulation expressing NK1.1 and CD27[11,22], while the $IL\text{-}17^+$ subpopulation expresses CCR6 and CD25[22]. Interestingly, γδ T cells have been shown to be the major source of IL-17 in different immune-mediated diseases, often producing much higher amounts of this cytokine than (conventional) $CD4^+$ Th17 cells, even if responding in similar or lower numbers than Th17 cells[23,24].

The functional role of γδ T cells during the pathogenesis of inflammatory disorders seems to be very diverse as they have been associated with pathogenic as well as protective functions, depending on the inflamed organ and disease model studied. In experimental glomerulonephritis, collagen-induced arthritis or experimental silicosis, for example, γδ T cells promote disease progression through production of IL-17[25-27]. In contrast, during adriamycin-induced nephropathy or concanavalin A-induced hepatitis, γδ T cells play a protective role through downregulation of the pathogenic functions of $CD4^+$ or NKT cells, respectively[20,28].

In recent years, a number of studies using material from patients with liver diseases as well as experimental models of liver injury revealed that γδ T cell subsets are altered during the progression of liver diseases, indicating that this unconventional lymphocyte population might be of utmost importance for determining the fate of inflammatory processes in the liver. In this review article, we aim to present and discuss the current knowledge about the functional role of γδ T cells and their subsets in the pathogenesis of liver disease in mice and humans, as well as possible mechanisms of their pro- or anti-inflammatory activities in the context of liver diseases (Table 1).

AUTOIMMUNE LIVER DISEASE

γδ T cells were already implicated in human autoimmune liver diseases two decades ago. Patients with primary sclerosing cholangitis or autoimmune hepatitis have been shown to display elevated numbers of γδ T cells in blood and liver when compared to healthy controls[29]. In the liver, γδ T cells were predominantly found in portal infiltrates and areas of bile duct proliferation or fibrogenesis, but the exact contribution of these cells to liver immunopathology remained elusive. Further insight into the functional role of γδ T cells in autoimmune hepatitis was provided more recently in a study of Zhao *et al*[20] by using the mouse model of concanavalin A (ConA)-induced fulminant hepatitis. This disease model of rapid liver inflammation and necrosis is dependent on the activation of $CD4^+$ T cells[30] and the role of IL-17 in this condition is controversially discussed (reviewed in[31]). In this study, the authors suggest a protective role of IL-17 produced by $V\gamma4^+$ γδ T cells through downregulation of the pathogenic function of NKT cells. NKT cells accumulate early after injury in the liver and promote the initiation of inflammatory responses and subsequent tissue damage by releasing pro-inflammatory cytokines[32]. $V\gamma4^+$ γδ T cells were the primary source of IL-17 in ConA-induced hepatitis and adoptive transfer of wild type (wt) γδ T cells was able to reduce the aggravated disease phenotype in γδ T cell deficient mice, associated with higher liver damage and IFNγ levels, to the level of wt mice. This function was critically dependent on IL-17 as this effect could not be observed when $TCR\delta^{-/-}$ mice were reconstituted with $IL\text{-}17^{-/-}$ γδ T cells[20]. These data indicate possible protective functions of $IL\text{-}17^+$ γδ T cells *via* NKT cell inhibition in immune-mediated liver diseases such as autoimmune hepatitis (Table 1).

VIRAL INFECTION

The essential role of T cell mediated immune responses

Table 1 Role of gamma-delta T cells in human and experimental liver disease

Species	Liver disease	TCR usage	Cytokine production	Other markers	Effector function(s)	Ref.
Protective functions of γδ T cells						
Mouse	Concanavalin A-induced hepatitis	Vγ4	IL-17		γδ T cells inhibit NKT cell function	[20]
Mouse	Experimental fibrosis	Vγ4?	IL-17, IL-22	CCR6, CD95L	γδ T cells induce stellate cell apoptosis and limit collagen production	[47]
Mouse	Listeria monocytogenes infection	Vγ4	IL-10		γδ T cells downregulate $CD8^+$ T cell effector function	[39]
		Vγ4/Vγ6	IL-17		γδ T cells are protective during early infection	[24]
Human	Liver metastasis of colon cancer	Vδ1	IFNγ, TNFα, IL-2	CD56, CD161	hepatic γδ T cells are cytotoxic against tumor cell lines in culture	[17]
Human	Pediatric tumor cell culture	Vγ9Vδ2	?		γδ T cells are cytotoxic against hepatoma cells in culture	[18]
Mouse	Adenoviral infection	Vγ4	IL-17		γδ T cells are critical for establishment of functional adaptive immune responses	[21]
Pathogenic functions of γδ T cells						
Mouse	*Schistosoma japonicum* infection	?	IL-17		γδ T cells contribute to immune-mediated pathology	[40]
Mouse	Adenoviral infection	?	IFN-γ	CXCR3	γδ T cells contribute to hepatocyte apoptosis *via* FasL engagement and recruitment of cytotoxic T cells	[37]
Mouse	MHV infection	?	TNF-α, IFN-γ, IL-17, IL-2	CD69, CD44	γδ T cells induce hepatocyte apoptosis via TNF-α-signaling	[38]
Human	HCV infection	Vδ1	IFN-γ	HLA-DR, CD95, CD45-RO	Activated γδ T cells contribute to HCV-mediated immunopathology	[19]

γδT cells: Gamma-delta T cells; IFN: Interferon; IL: Interleukin; TNF: Tumor necrosis factor; MHV: Mouse hepatitis virus; HCV: Hepatitis C virus.

in either clearing viral hepatitis or allowing persistent chronic infections is well established[33]. However, less data exist on γδ T cells in hepatitis B or C. In patients with chronic hepatitis B virus (HBV) infection, intrahepatic as well as peripheral γδ T cell numbers inversely correlate with disease severity[34]. Wu *et al*[34] showed that mainly $Vδ2^+$ γδ T cells are reduced and that these cells display an effector-memory phenotype with expression of CD45RA, MHC class Ⅱ molecule human leukocyte antigens (HLA)-DR and CD38. Furthermore, these cells produce high levels of IFNγ but not IL-17 and are able to inhibit cytokine production of pathogenic $CD4^+$ Th17 cells through cell contact- as well as IFNγ-dependent mechanisms. Therefore, the authors concluded that reduced numbers of γδ T cells account for decreased inhibition of Th17 cells, resulting in higher liver damage and pathology.

In contrast, several studies have shown that γδ T cells are enriched in the livers of patients with chronic hepatitis C virus (HCV) infection when compared to healthy controls or peripheral blood[19,35,36]. Agrati and colleagues demonstrated that these γδ T cells are predominantly $Vδ1^+$ and display an effector-memory phenotype as they express HLA-DR and CD95[19]. These cells also produce increased levels of IFNγ during HCV infection and therefore very likely contribute to HCV-induced immunopathology in the liver. Furthermore, an additional study by Tseng *et al*[36] showed that γδ T cells isolated from livers of HCV patients are cytotoxic against primary human hepatocytes in culture, suggesting that γδ T cells might contribute to HCV-triggered liver injury.

A similar effect is seen in mice with adenoviral infection. IFN-γ-producing γδ T cells accumulate around infected hepatocytes and contribute to hepatocyte death through Fas-mediated apoptosis[37]. Furthermore, IFNγ production induces the release of chemokines like CXCL9 by hepatocytes, which further recruits γδ T cells and $CD8^+$ cytotoxic T cells. The importance of γδ T cells for these pathogenic processes is underlined by the fact that γδ T cell deficient mice are protected from adenovirus-induced liver injury. However, these mice show no difference in viral clearance. Another study by Hou *et al*[21] shows that IL-17 producing γδ T cells also increase in adenovirus-infected murine liver. Consistent with the results obtained in ConA-induced hepatitis, $Vγ4^+$ γδ T cells are the major IL-17 producers and IL-17 secretion by these cells is critical for the development of a functional antiviral immune response and subsequent clearance of the virus.

In mouse hepatitis virus (MHV) infection, γδ T cells play a clearly pathogenic role but *via* a different mechanism[38]. Although IFNγ- and IL-17- producing γδ T cells accumulate in the liver also in this model, their function seems to be rather dependent on tumor necrosis factor (TNF) α-production. Activated hepatic γδ T cells are cytotoxic against MHV infected hepatocytes but this effect does not require cell-cell contact or IFNγ-/IL-17-signaling, while blockade of TNFα leads to markedly reduced hepatocytotoxicity[38].

Taken together, the functional role of γδ T cells during viral infection of the liver seems to be highly dependent on the subset involved. While $Vδ1^+$ and $Vδ2^+$ T cells are associated with production of IFNγ and progression of liver immunopathology, the $Vγ4^+$ IL-17 producing subset of γδ T cells seems to be rather important for viral clearance. The fact that liver injury during MHV infection is dependent on TNF-α production by γδ T cells might suggest that a third subset of γδ T cells is functionally involved in viral-induced liver diseases.

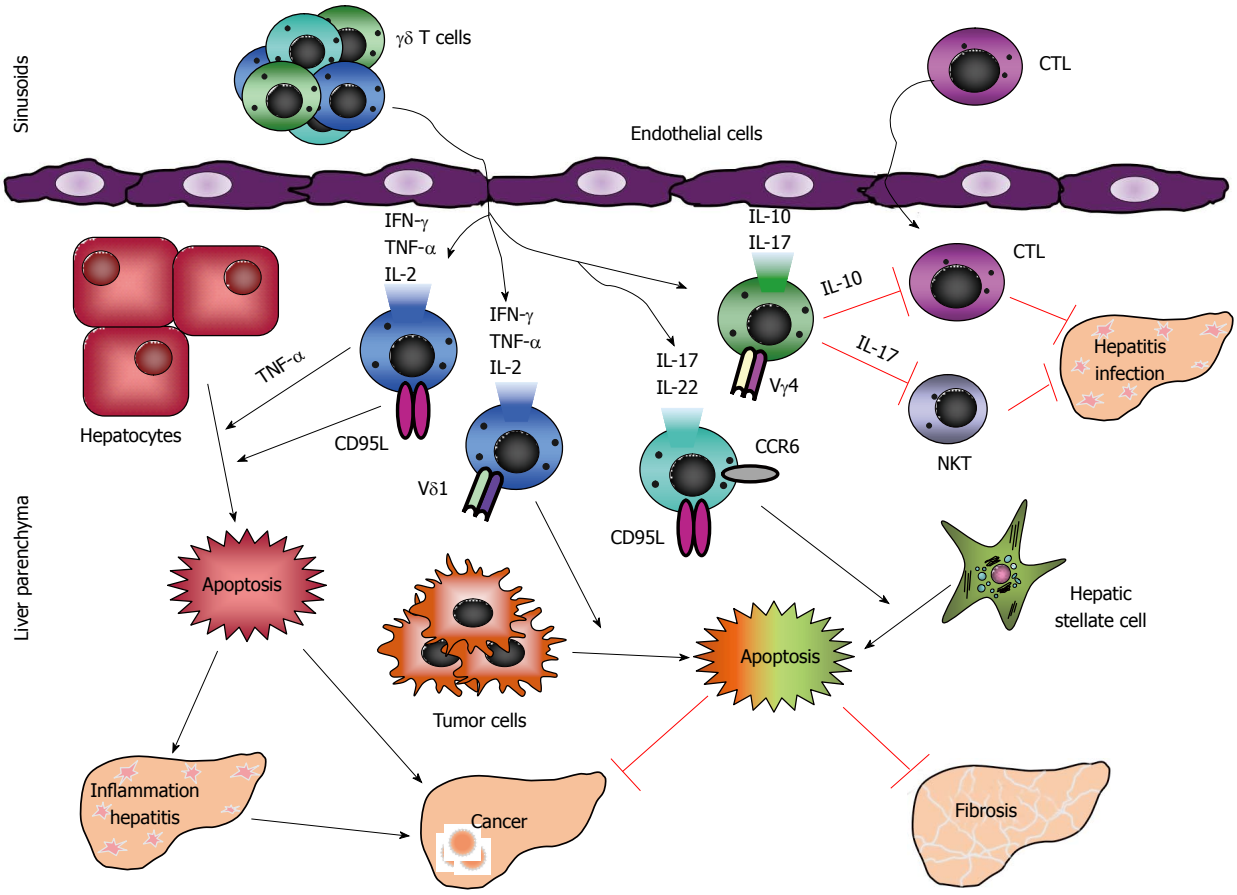

Figure 1 Role of gamma-delta T cells in liver disease. Upon liver damage several subsets of gamma-delta (γδ) T cells are recruited to the liver, where they can exert different functions on numerous cell types, ultimately resulting in protective or pathogenic effects on the outcome of liver disease. Pathogenic effects include induction of hepatocyte apoptosis by interferon (IFN)γ - and/or tumor necrosis factor (TNF) α-producing γδ T cells, mediated via death receptor signaling (TNF receptors or Fas/CD95). However, the Vδ1$^+$ IFNγ-producing subset can also have beneficial functions as they drive tumor cells apoptosis. Other protective functions can be attributed to Vγ4$^+$ T cells, which produce interleukin (IL)-17 and IL-10, and can downregulate pathogenic effector functions of other lymphocytes like natural killer T (NKT) cells or cytotoxic T cells, respectively. IL-17$^+$ γδ T cells have also been shown to induce Fas-mediated apoptosis of hepatic stellate cells (the main producer of collagen during hepatofibrogenesis), thereby limiting liver fibrosis.

BACTERIAL AND PARASITIC LIVER INFECTIONS

γδ T cells have been shown to exert protective functions in bacterial infections of the liver. γδ T cell deficient mice infected with *Listeria monocytogenes* develop increased liver pathology which is caused by infiltrating CD8$^+$ T cells producing high levels of TNF-α[39]. This pathogenic effect can be prevented through adoptive transfer of Vγ4$^+$ γδ T cells. These cells produce high levels of IL-10, which in turn downregulates TNF-α production in CD8$^+$ T cells (Figure 1). Furthermore, Vγ4$^+$ T cells are also the major IL-17 producing cell type during *Listeria* infection and γδ T cell-derived IL-17 is critically needed for protective immunity during early infection[24]. IL-17 deficient mice reconstituted with γδ T cell-deficient bone marrow, meaning that γδ T cells are able to produce IL-17 but γδ T cells are not, show a much higher bacterial burden in the liver than mice reconstituted with wt bone marrow[24]. In contrast, during *Schistosoma japonicum* infection IL-17 production by γδ T cells seems to have a more pathogenic role[40]. Although γδ T cells are the major IL-17 producing cell type also in this model, neutralization of IL-17 reduced liver inflammation and pathology in this case.

During malaria infection, however, γδ T cells play only a minor role as long as conventional adaptive T cell responses are intact, demonstrated by the fact that γδ T cell deficient mice survive plasmodium infection without extensive organ failure[41]. γδ T cells are needed for protective immunity against the parasite only in mice deficient for γδ T cells. In this case, depletion of γδ T cells leads to severe immunopathology because development of the parasite is not inhibited, an effect that can be reversed through adoptive transfer of γδ T cells[41].

As described above, γδ T cells can have opposing effects in different infection models. This further underlines the functional heterogeneity of the different γδ T cell subsets distinguished by cytokine production or usage of specific receptor chains. The impact that γδ T cells have on the outcome of different infectious diseases might also be influenced by the nature of the adaptive immune response induced by the microorganism itself as this could change the local cytokine milieu dramatically.

LIVER FIBROSIS

Independent from the underlying etiology of liver disease, such as viral hepatitis, alcoholic and non-alcoholic steatohepatitis or other origins, chronic liver diseases characteristically progress from tissue injury to chronic hepatitis and fibrosis to liver cirrhosis as the end-stage of chronic liver diseases[42]. Persistent inflammation in the liver is considered the driving force for disease progression. Over recent years, several studies have emphasized the crucial role of various immune cell subsets for controlling inflammation and fibrogenesis in the liver and the interplay between the different leukocyte populations, including monocytes, Kupffer cells, NK/NKT or T lymphocytes, appears to be tightly regulated by cytokines and chemokines[43,44]. Although IL-17 has been recognized as an important regulatory cytokine in hepatic inflammation[31], relatively few data exist on the contribution of γδ T cells to the pathogenesis of liver fibrosis. γδ T cells accumulate in fibrotic liver and contribute to IL-17 production in different experimental models of chronic liver injury, as well as liver samples of patients with chronic hepatitis[45,46]. Interestingly, IL-17 itself, produced mainly by αβ T cells and neutrophils, was found to promote fibrosis progression through activation of hepatic stellate cells (HSC) and Kupffer cells.

In contrast, hepatic γδ T cells can be associated with protective functions in murine chronic liver injury but these functions appear to be independent from the signature cytokine IL-17. We recently showed that specifically the CCR6 expressing subtype of γδ T cells, producing IL-17 and IL-22, accumulates in fibrotic livers of mice subjected to experimental liver injury models[47]. These cells are capable of limiting fibrosis progression through induction of apoptosis in HSC, the major collagen producing cell type in the liver. Nevertheless, this effect does not depend on their IL-17 or IL-22 production but is rather mediated through Fas/Fas-ligand (FasL) interactions. IL-17 deficient γδ T cells are able to limit liver fibrogenesis to the same extent as wt γδ T cells and blockade of IL-22 could not reduce HSC apoptosis, while use of a FasL-blocking antibody significantly inhibited HSC apoptosis (Figure 1). Thus, these data indicate that γδ T cells, at least its CCR6 expressing subset, represent an important anti-fibrotic pathway in hepatic inflammation by ameliorating the inflammatory reaction and the activation of collagen-producing stellate cells in chronically injured liver.

LIVER CANCER

More than two decades ago the first studies showed that γδ T cells accumulate in tumor bearing liver. Patients with hepatic malignancies as well as tumor bearing mice show elevated levels of γδ T cells in the liver when compared to healthy controls[17,48]. Usually these cells display an activated phenotype with expression of CD56, CD161 and LFA-1 and are cytotoxic against hepatoma cells and Daudi targets in culture[17,18]. Furthermore, murine Vδ1$^+$ γδ T cells induced in response to cytomegalovirus (CMV) infection have been shown to inhibit development of liver metastases in a colon cancer model[49]. These findings suggest that γδ T cells might contribute to antitumoral immune responses, likely by promoting direct cytotoxic responses to malignant parenchymal cells (Figure 1). However, tumor cells can escape γδ T cell responses through downregulation of the respective ligands[18].

Although detailed mechanistical studies on anti-tumoral responses of γδ T cells in the liver are still lacking, further insight into these mechanism might be provided by a recent study on recruitment of γδ T cells in the B16 melanoma model[50]. In this model, γδ T cells inhibit tumor growth as γδ T cell-deficient mice develop larger tumors than their wild type counterparts. A similar effect is seen in CCR2- as well as CCL2-deficient mice, which display reduced γδ T cell infiltrates in B16 lesion and a higher tumor growth rate. Moreover, this study also shows that murine as well as human peripheral γδ T cells migrate toward CCL2 *in vitro*[50]. Since this effect could only be observed with Vδ1$^+$ but not Vδ2$^+$ γδ T cells, this mechanism might very well also play a role in hepatic malignancies.

CONCLUSION

γδ T cells have been shown to accumulate in the liver upon various inflammatory conditions which lead to hepatic fibrosis and other types of immunopathology when becoming chronic. The exact contribution of these lymphocytes to liver inflammation seems to be highly dependent on the subsets involved, which can be identified by the specific cytokines they produce and their expression of different T cell receptor chains. γδ T cells producing IFNγ often co-express TNFα and the Vδ1 chain but usually do not produce IL-17, which is often co-expressed with Vγ4 chains. The effect of these subsets on the outcome of liver disease also depends in part on the underlying liver disease etiology. Accordingly, the IFNγ$^+$ subset is able to induce apoptosis in different cell types, which might have pathogenic or beneficial effects on liver immunopathology depending on whether hepatocytes or tumor cells are affected. In contrast, IL-17 producing γδ T cells are often associated with protective functions in liver inflammation as they can inhibit pathogenic effector functions of cytotoxic T cells or NKT cells, as well as limit hepatofibrogenesis through inhibition of hepatic stellate cells. Nevertheless, the results obtained in human liver disease as well as murine models are not fully conclusive at present as many studies lack detailed analysis on the correlation of cytokine production with specific surface markers such as TCR chains. Therefore, it is not clear whether the diverse functions that γδ T cells have during different liver diseases are executed by very few subsets according to the cytokines they produce or by a huge variety of γδ T cells with redundant cytokine profiles. Thus, it is of utmost importance to further define γδ T cell subsets in acute and chronic liver inflammation as well as the cytokines they produce in order to assess

whether interference with γδ T cells might be useful as a therapeutic target for the treatment of liver disease.

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RESEARCH REPORT

Liver biopsy: Analysis of results of two specialist teams

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Abstract

AIM: To analyze the safety and the adequacy of a sample of liver biopsies (LB) obtained by gastroenterologist (G) and interventional radiologist (IR) teams.

METHODS: Medical records of consecutive patients evaluated at our GI unit from 01/01/2004 to 31/12/2010 for whom LB was considered necessary to diagnose and/or stage liver disease, both in the setting of day hospital and regular admission (RA) care, were retrieved and the data entered in a database. Patients were divided into two groups: one undergoing an ultrasonography (US)-assisted procedure by the G team and one undergoing US-guided biopsy by the IR team. For the first group, an intercostal approach (US-assisted) and a Menghini modified type needle 16 G (length 90 mm) were used. The IR team used a subcostal approach (US-guided) and a semiautomatic modified Menghini type needle 18 G (length 150 mm). All the biopsies were evaluated for appropriateness according to the current guidelines. The number of portal tracts present in each biopsy was assessed by a revision performed by a single pathologist unaware of the previous pathology report. Clinical, laboratory and demographic patient characteristics, the adverse events rate and the diagnostic adequacy of LB were analyzed.

RESULTS: During the study period, 226 patients, 126 males (56%) and 100 females (44%), underwent LB: 167 (74%) were carried out by the G team, whereas 59 (26%) by the IR team. LB was mostly performed in a day hospital setting by the G team, while IR completed more procedures on inpatients ($P < 0.0001$). The groups did not differ in median age, body mass index (BMI), presence of comorbidities and coagulation parameters. Complications occurred in 26 patients (16 G team *vs* 10 IR team, $P = 0.15$). Most gross samples obtained were considered suitable for basal histological evaluation, with no difference among the two teams (96.4% G team *vs* 91.5% IR, $P = 0.16$). However, the samples obtained by the G team had a higher mean number of portal tracts (G team 9.5 ± 4.8; range 1-29 *vs* IR team 7.8 ± 4.1; range 1-20) ($P = 0.0192$) and a longer mean length (G team 22 mm ± 8.8 *vs* IR team 15 ± 6.5 mm) ($P = 0.0001$).

CONCLUSION: LB can be performed with similar outcomes both by G and IR. Use of larger dimension needles allows obtaining better samples, with a similar

rate of adverse events.

Key words: Liver biopsy; Ultrasound-guided biopsy; Ultrasound-assisted biopsy; Menghini needle; Sample adequacy; Portal tracts.

Core tip: Gastroenterologists and interventional radiologists are equally proficient in performing liver biopsy, both in a day hospital and regular admission setting, even with different techniques used (ultrasound-guided and ultrasound-assisted). However, a biopsy performed with larger needles provides better samples for histopathological evaluation, with no increase of morbidity or mortality rates compared to those obtained using needles of smaller size.

Anania G, Gigante E, Piciucchi M, Pilozzi E, Pucci E, Pellicelli AM, Capotondi C, Rossi M, Baccini F, Antonelli G, Begini P, Delle Fave G, Marignani M. Liver biopsy: Analysis of results of two specialist teams. *World J Gastrointest Pathophysiol* 2014; 5(2): 114-119 Available from: URL: http://www.wjgnet.com/2150-5330/full/v5/i2/114.htm DOI: http://dx.doi.org/10.4291/wjgp.v5.i2.114

INTRODUCTION

Liver biopsy is an invasive procedure aimed at obtaining a sample of liver tissue for the evaluation of acute and chronic liver disease[1]. Sampling can be performed either during surgery or by percutaneous needle biopsy using different techniques[2]. Currently, this procedure is supported by imaging techniques, such as ultrasonography (US) or computed tomography, with a significant reduction of complications[3-5].

Our study aimed to analyze the results of the same medical-surgical procedure, percutaneous liver biopsies (LB), performed by two different medical teams: gastroenterologists (G) and interventional radiologists (IR). The G team performs the procedure with the US-assisted method (the area in which to insert the needle is identified with US before LB) *via* an intercostal approach, while the IR team performs the procedure with a US-guided technique (LB is performed by the operator during US, sometimes with a needle supported and directed by a dedicated US probe) with a subcostal approach[6,7].

There are presently no comparative data available on these two different modalities of LB performance. The two approaches were compared, analyzing the characteristics of patients undergoing LB, safety of the procedure, and capability of providing suitable material for histopathological evaluation.

MATERIALS AND METHODS

Medical records of consecutive patients evaluated at our GI unit, from 01/01/2004 to 31/12/2010, and for whom LB was considered necessary to diagnose and/or stage liver disease, both in the setting of day hospital (DH) and regular admission (RA) care, were retrieved and the data entered in a database. Indications to undergo LB were those provided by the main international guidelines[2]. Patients were divided into 2 groups: one undergoing a US-assisted procedure by the G team and one undergoing a US-guided biopsy by the IR team. For the first group, an intercostal approach (US-assisted) and a Menghini modified type needle 16 G (length 90 mm) were used. For the second group, the IR team used a subcostal approach (US-guided) and a semiautomatic modified Menghini type needle 18G (length 150 mm)[6,7] (Table 1). The condition of the patients was monitored with subsequent blood pressure and complete blood count testing at two and four hours post-procedure[8,9]. A telephone follow up call was made a week after the procedure in order to detect possible late adverse events/complications.

Table 1 Details of the techniques adopted for liver biopsy by the two teams

	Gastroenterology team	Interventional radiology team
Needle characteristics	Menghini modified type needle 16 G (9 cm)	Menghini type needle semiautomatic, modified 18 G (15 cm)
Method	US-assisted	US-guided
Approach	Intercostal	Subcostal

US: Ultrasonography.

All the biopsies were evaluated for appropriateness according to the current guidelines by a team of pathologists experienced in the evaluation of liver parenchyma at our hospital. All specimens were fixed in formalin, embedded in paraffin and sectioned by microtome. Specimens were routinely stained with hematoxylin and eosin. The adequate specimen for diagnosis was considered to have a length between 1-4 cm[2]. The number of portal tracts present in each biopsy was assessed by a revision performed by a single pathologist unaware of the previous pathology report. The portal tracts were identified by the presence of foci of connective tissue and at least two luminal structures embedded in the connective tissue and their number counted and entered in a database. The presence of at least 6 portal tracts was used to define an optimal sample.

Clinical, laboratory and demographic characteristics of the study patients, adverse events rate and diagnostic adequacy of LB were analyzed by the Student's t test for continuous variables and by Fisher's exact test in case of binary variables (Table 2). Data are expressed as percentage (number/total), median (range) for demographic and laboratory data, and as mean ± SD for number of portal tract per bioptic sample and length of samples.

All patients gave informed consent for the use of clinical data at the time of admission.

RESULTS

During the study period, 365 patients underwent liver

Table 2 Patient characteristics in the two groups

Group	Team G	Team IR	*P*
Male sex, % (number/total)	60% (101/167)	42% (25/59)	0.021
AGE, years, median (range)	50.5 (16-70)	52 (19-73)	0.41
BMI, median (range)	24 (17-36)	24 (18-41)	0.94
PLATELETS/mm^3, median (range)	199000 (77000-797000)	204000 (65000-394000)	0.65
INR, median (range)	1 (0.86-1.44)	1.02 (0.87-1.94)	0.24
Complications, % (number/total)	9.5% (16/167)	17% (10/59)	0.15

M: Male; BMI: Body mass index; INR: International normalized ratio; IR: Interventional radiology.

Table 3 Occurrence of adverse events following liver biopsy by setting and team performing the procedure

	Regular admission	Day hospital	Team G	Team IR
Total number of adverse events	13	13	16	10
Pain moderate to severe % (number/total)	77% (10/13)	70% (9/13)	68% (11/16)	80% (8/10)
Relevant biochemical abnormalities[1] % (number/total)	15% (2/13)	31% (4/13)	25% (4/16)	20% (2/10)
Nausea/vomiting % (number/total)	7% (1/13)	(0/13)	6% (1/16)	(0/10)

[1]Mild increase of white blood cells (4 cases); mild hemoglobin decrease < 2 mg/dL from baseline (1 case); thrombocytopenia (1 case). IR: Interventional radiology.

biopsy at our center. From this group, those who had a LB to investigate liver mass lesions were excluded (n = 139, 38%). The remaining 226 patients (62%) underwent LB to evaluate liver parenchyma. Of these 226 patients [126 males (56%), 100 females (44%)], 167 (74%) underwent LB performed by the G team (intercostal approach, US-assisted) and 59 (26%) by the IR team (subcostal approach, US-guided). The hospital setting in which LB was performed was significantly different between the two groups: RA= 29% (48/167) and DH = 71% (119/167) for the G team *vs* RA = 64% (38/59) and DH = 36% (21/59) for the IR team ($P < 0.0001$). The approach was intercostal in all 167 patients by the G team and subcostal in all 59 managed by the IR team. The G team performed LB in a slightly but significantly higher number of male patients with no differences in median age of patients in the two groups observed (Table 2). Median value of body mass index (BMI) was also similar in both groups (Table 2). Fifty-two patients (23%) were affected by significant comorbidities with no significant differences between the two groups. Similarly, median international normalized ratio and platelet concentration were not significantly different in the two groups (Table 2). The most frequent indication for LB was staging and grading liver disease caused by viral hepatitis B and C. In fact, out of a total of 226 patients, 141 (62%) had chronic viral infection, 23% of whom were affected by hepatitis B (32/141) and 77% (109/141) by hepatitis C. There were 26 complications in as many patients (11.5%, 26/226). No difference in terms of incidence of complications was observed between the two teams (G team: 9.5%, 16/167; IR team: 17%, 10/59, P = 0.15) despite the different needles and approaches used. It was not necessary to convert to RA in any of the cases of adverse events occurring in patients undergoing LB in the DH setting. We also performed a subgroup analysis of the rate of adverse events observed in the RA setting and no difference in the G (6/48) *vs* IR (7/38) team was shown (P = 0.548). Subgroup analysis performed on the rate of adverse events observed in the DH setting also did not show any significant difference between the two groups, G (10/119) *vs* IR (3/21) (P = 0.413). The adverse events that occurred are summarized in Table 3. Telephonic surveillance at one week after the procedure was negative in all cases discharged without complications after LB.

Table 4 Characteristics of bioptic samples

Number of bioptic samples	G Team 167	IR Team 59	*P* = NA
Samples adequate for diagnosis % (number/total)	96.4% (161/167)	91.5% (54/59)	0.16
Sample length[1] mean ± SD	22 mm ± 8.8	15 mm ± 6.5	< 0.0001
Number of portal tract per sample[2] mean ± SD	9.5 ± 4.8	7.8 ± 4.1	0.0192

[1]Evaluation performed on 215 (161 by G team, 54 by IR), considered to be adequate for diagnosis; [2]Evaluation performed on 205 samples (151 by G team, 54 by IR). NA: not applicable; IR: Interventional radiology.

The overall number of LB samples not suitable for histological evaluation was low (11/226, 4.9%) and there was no statistical difference in the number of suitable and unsuitable samples obtained by the two teams (Table 4). Data on the number of portal tracts per bioptic sample were evaluable for 205 biopsies, 151 performed by the G team and 54 by the IR team respectively. At the time of retrospective re-evaluation of bioptic samples for portal tract count, 10 samples, all from the G team, were no longer available. Interestingly, samples provided by the G team had a significantly higher number of portal tracts compared to those obtained by the IR team (Table 3; P = 0.0192). Overall, 30.7% (63/205) of bioptic samples had ⩾ 11 complete portal tracts, 34% (52/151) and 20% (11/54) G *vs* IR respectively. Bioptic samples with ⩾ 6 complete portal tracts were overall 76.6% (157/205), 78.1% (118/151) and 72.2% (39/54) G *vs* IR respectively. Moreover, the samples obtained by the G team were longer compared with those of the IR team (Table 4; P = 0.0001).

DISCUSSION

There are few studies comparing the outcomes of LB

on parenchyma adopting different approaches (subcostal versus intercostal) and different imaging modalities to aid its performance (US-guided *vs* US-assisted)[7,10]. Thus, the results of our study add information to the available literature. From our data, it emerges that both LB performance modalities, supported and implemented by the use of US, allow achieving optimal results in terms of patient safety. These data are not present in the literature, which has been mainly focused on the comparison of US *vs* non-US-guided procedures[2,11-13].

In addition, even with the limitations inherent to the retrospective nature of our analysis, since the patients had similar coagulative profiles, BMI and prevalence of comorbidities, there were no elements suggesting a preferential choice of one team over the other. The main reason that guided the choice of one team over the other was the availability of either team at the time the procedure was ordered.

Our results also show that the two groups are homogeneous regarding the occurrence of complications (9.5% *vs* 17%, $P = 0.15$) and that in all occurrences there was no increased morbidity, such as a requirement for surgery, blood transfusions and IR treatments, or death (mortality). Also, no complications that occurred in patients managed in DH led to the conversion to RA, further supporting the current data regarding the safety of LB[2,14].

Unfortunately, the smaller number of procedures performed by IR might lead to underestimating the difference between the two groups, an intrinsic bias of the retrospective nature of this study, which in turn limits the power of data analysis. It has to be pointed out that in our study, localized pain at the site of needle insertion was also defined as a complication and that this contributed to more than 73% of all complications, a figure well within those reported in the literature (up to 84%)[2,11,14-17]. This event is so common that some authors do not even include it among the complications. Thus, we performed a sub-analysis separating the adverse event pain from the other signs and symptoms that developed after the performance of LB. Again, no differences were observed between the results obtained by the G and the IR team ($P = 1$).

Apart from pain, the most common adverse events were biochemical abnormalities such as a mild increased white blood cell count and a mild hemoglobin decrease (< 2 gm/dL) from baseline, observed in a marginal number of patients (Table 3). This absence of difference is interesting since higher percentages of complications have been reported when larger needles are used, as for the G team. Thus, performance of LB in a DH setting confirms its safety, with the post procedure monitoring protocol allowing safe discharge of patients after brief observation (4 h) and with the negative telephonic surveillance performed one week after the procedure integrating these safety data. This approach contributes to containing hospital costs by reducing the need for admission to perform this procedure. In addition, considering a health service system based on a disease related group reimbursement such ours, ordering LB to a service or department not belonging to the one which has posed the indication for it has many potential positive aspects. Firstly, it is obviously less expensive since it uses resources already available to the unit ordering the procedure and secondly, it does reduce the burden of this relatively simple procedure to the already busy schedule of the IR team, without encumbering their high technology and expensive wards. Thus, being equally safe and possibly less expensive, LB should preferably be performed in-house in the gastroenterology department[18,19].

Our results also show that even if the adequacy of samples obtained by the two teams are comparable in terms of overall dimension, the bigger needle used by the G team provided a larger number of evaluable portal tracts and sample length, a necessary requirement for better histopathological evaluation, as previously demonstrated[20-22].

A further possible limitation of our data is represented by the percentage of samples with a number ⩾ 11 of complete portal tracts (30.7%). As suggested by the 2009 AASLD guidelines, the presence of < 11 complete portal tracts should be noted in the pathology report, with recognition that diagnosis, grading and staging may be incorrect due to an insufficient sample size. Nevertheless, the presence of 6 portal tracts has previously been considered to be acceptable for diagnosis[23] and overall, 76.6% (157/205) of samples obtained in our study were above this limit. Thus, since we have chosen the latter numeric parameter, we acknowledge that the reduced number of portal tracts obtained might have affected the accuracy of diagnosis. However, the significantly higher mean number of portal tracts obtained by the biopsy samples performed by the G team suggests a higher opportunity for better diagnostic findings.

Interestingly, even if intuitively a bigger needle should obtain a bigger sample and consequently a higher number of portal tracts, available evidence is at times contrasting. In fact, a systematic review by Cholongitas *et al*[24] described that LB performed with bigger needles did obtain a slightly higher number of portal tracts and samples of longer length but that these differences did not reach statistical significance. On the other hand, data from other authors obtained in a single center study also suggested that the use of a bigger needle (16 G as in our case for the G team) can obtain samples with a significantly higher number of portal tracts[25,26]. Considering that the use of a 16 G needle is also suggested by AASLD guidelines to obtain LB 3 cm long and to avoid sampling errors, especially for diffuse parenchymal diseases such as cirrhosis, we concluded that our data provide further support to the use of a biopsy needle of larger gauge to perform LB in terms of sample adequacy, with a comparable incidence of complications[2].

Thus, our retrospective, single center study suggests that LB can be performed with equal safety with different techniques performed by specialists from different units. At the same time, the better performance in terms of sample adequacy obtained by needles of larger gauge

also suggests their use. Cost effectiveness analyses are needed to better define the economic burden inherent to the different approaches.

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COMMENTS

Background

Percutaneous liver biopsy is a pivotal diagnostic procedure in the management of liver diseases. In order to support the diagnosis process, an adequate sample of tissue is required. Several different technical approaches and devices have been developed and are available.

Research frontiers

Presently, percutaneous liver biopsies are carried out with the assistance of imaging techniques such as ultrasonography, with an ultrasonography (US)-assisted or US-guided technique. Furthermore, a wide range of needle sizes are used and the choice of one technique or needle over the other is mainly based on physician experience. To date, there are just a few comparative studies on this matter.

Innovations and breakthroughs

In previous studies, the use of bigger needles to perform liver biopsies was not univocally associated with more suitable samples, thus authros performed the analysis to confirm that the use of a bigger needle could provide more proficient biopsies with a similar safety profile.

Applications

The study results suggest that the use of bigger needles could supply more useful liver samples with a similar incidence of adverse events.

Peer review

Anania *et al* propose an interesting study comparing the parameters of two approaches of liver biopsy, US assisted and US guided, performed by two teams, one of gastroenterologists and one of interventional radiologists. The article has a very interesting idea behind it.

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Controversial issues regarding the roles of IL-10 and IFN-γ in active/inactive chronic hepatitis B

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Abstract

According to the important roles played by cytokines in induction of appropriate immune responses against hepatitis B virus (HBV), Dimitropoulou *et al* have examined the important cytokines in their patients. They showed that the serum levels of interleukin 10 (IL-10) and interferon-γ (IFN-γ) were decreased in patients with HBeAg-negative chronic active hepatitis B compared with the inactive hepatitis B virus carriers (Dimitropoulou *et al* 2013). The controversy can be considered regarding the decreased serum levels of IFN-γ in the HBeAg-negative chronic active hepatitis B patients. They concluded that subsequent to decreased expression of IFN-γ, the process of HBV proliferation led to liver diseases. Previous studies stated that HBV is not directly cytopathic for the infected hepatocytes and immune responses are the main reason for destruction of hepatocytes (Chisari *et al*, 2010). Scientists believe that immune responses against HBV are stronger in active forms of chronic HBV infected patients than inactive forms (Zhang *et al*, 2012). Therefore, the findings from Dimitropoulou *et al* may deserve further attention and discussion. Additionally, downregulation of IL-10 in chronically active hepatitis B infected patients has also confirmed our claim. IL-10 is an anti-inflammatory cytokine and its expression is increased in inactive forms in order to downregulate immune responses (Arababadi *et al*, 2012). Thus, based on the results from Dimitropoulou *et al*, it can be concluded that increased immune responses in chronically active hepatitis B infected patients are related to declined expression of IL-10 and interestingly IFN-γ is not involved in induction of immune responses in these patients.

Key words: Hepatitis B virus; Interferon-γ; Interleukin-10

Core tip: Cytokines play a central role in the induction of appropriate immune responses against hepatitis B, as well as the clinical manifestations of the disease. Dimitropoulou *et al* showed that serum levels of interleukin 10 and interferon-γ decreased in patients with HBeAg-negative chronic active hepatitis B compared with inactive hepatitis B virus (HBV) carriers (Dimitropoulou *et al*, 2013) and concluded that this can lead to liver disease. However, we challenge their conclusion because we believe that inappropriate host immune responses are the main causes responsible for the clinical manifestations of the disease, but not the actual replication of the HBV particles.

Khorramdelazad H, Hassanshahi G, Arababadi MK. Controversial issues regarding the roles of IL-10 and IFN-γ in active/inactive chronic hepatitis B. *World J Gastrointest Pathophysiol* 2014; 5(2): 120-121 Available from: URL: http://www.wjgnet.com/2150-5330/full/v5/i2/120.htm DOI: http://dx.doi.org/10.4291/wjgp.v5.i2.120

TO THE EDITOR

We have carefully reviewed the article by Dimitropoulou

et al[1] who examined the serum levels of both pro-and anti-inflammatory cytokines in patients with hepatitis B e antigen (HBeAg)-negative chronic active hepatitis B and inactive hepatitis B virus (HBV) carriers. It is well established that the serum levels of cytokines change during various clinical presentations of hepatitis B[2,3]. Based on the important roles played by cytokines in the induction of appropriate immune responses against HBV, Dimitropoulou *et al*[1] examined the most relevant cytokines in hepatitis B infected patients. They reported that the serum levels of interleukin-10 (IL-10) and interferon-γ (IFN-γ) were decreased in patients with HBeAg-negative chronic active hepatitis B compared with inactive hepatitis B virus carriers.

The apparent controversy arises from the author's discussion regarding decreased serum levels of IFN-γ in the HBeAg-negative chronic active hepatitis B patients. The authors have concluded that subsequent to decreased expression of IFN-γ, the processes of HBV proliferation can lead to liver diseases. Previous studies have demonstrated that HBV is not directly cytopathic to the infected hepatocytes and that the main destruction of hepatocytes is caused by host immune responses[4]. Researchers believe that immune responses against HBV are stronger in active forms of chronically HBV infected patients as opposed to the inactive forms[5]. Therefore, the discussion addressing these observations should be carefully reviewed, even for a revision. Additionally, downregulation of IL-10 in chronically active hepatitis B infected patients also confirms our claim. IL-10 is an anti-inflammatory cytokine and its expression is increased in inactive forms in order to attenuate immune responses[2]. Thus, based on the results presented by Dimitropoulou *et al*[1] it can be concluded that increased immune responses in chronically active hepatitis B infected patients is related to reduced expression of IL-10 and interestingly IFN-γ is not involved in the induction of immune responses in these patients.

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TOPIC HIGHLIGHT

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AIM AND SCOPE

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TOPIC HIGHLIGHT

WJGP 5th Anniversary Special Issues (1): *Helicobacter pylori*

Biofilms and *Helicobacter pylori*: Dissemination and persistence within the environment and host

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Key words: *Helicobacter pylori*; Biofilm; Coccoid forms; Virulence; Water

Core tip: The ability of *Helicobacter pylori* (*H. pylori*) to form biofilms is fundamental to its pathogenicity. Research into the mechanisms behind *H. pylori* resuscitation from coccoid to virulent spiral forms will aid a better understanding into infection recurrence in the host and the external environment.

Percival SL, Suleman L. Biofilms and *Helicobacter pylori:* Dissemination and persistence within the environment and host. *World J Gastrointest Pathophysiol* 2014; 5(3): 122-132 Available from: URL: http://www.wjgnet.com/2150-5330/full/v5/i3/122.htm DOI: http://dx.doi.org/10.4291/wjgp.v5.i3.122

Abstract

The presence of viable *Helicobacter pylori* (*H. pylori*) in the environment is considered to contribute to the levels of *H. pylori* found in the human population, which also aids to increase its genetic variability and its environmental adaptability and persistence. *H. pylori* form biofilms both within the *in vitro* and *in vivo* environment. This represents an important attribute that assists the survival of this bacterium within environments that are both hostile and adverse to proliferation. It is the aim of this paper to review the ability of *H. pylori* to form biofilms *in vivo* and *in vitro* and to address the inherent mechanisms considered to significantly enhance its persistence within the host and in external environments. Furthermore, the dissemination of *H. pylori* in the external environment and within the human body and its impact upon infection control will be discussed.

INTRODUCTION

Helicobacter pylori (*H. pylori*) is an opportunistic pathogen that plays an important role in the aetiology of peptic and gastric ulcers. *H. pylori* primarily colonizes the antral part of the stomach whereby they either adhere to the walls of the stomach or simply remain in a planktonic, free-floating state. This bacterium has been reported to spread from the stomach to the intestine where it is then secreted in faeces[1]. *H. pylori* infection is known to be associated with nausea and vomiting which can lead to the spread of this pathogen to the oral cavity, leading to the colonisation of gingival and dental plaques[2].

H. pylori has been reported to colonise over half the world's population with clinical signs of infection only manifesting in less than 20% of these individuals[3]. Nevertheless, the majority of these individuals are colonised with *H. pylori* for life unless eradication using appropriate chemotherapeutic agents is successful. Lifelong colonisation seems to be due to the ability of some strains

of *H. pylori* to both adapt to the host's immunological responses and to also withstand the constantly changing gastric environment. In genetically predisposed individuals, colonisation with *H. pylori* is reported to heighten the risk of developing cancer[4].

H. pylori can be described as Gram negative, spiral- (S-shape) or cocci-shaped bacteria. It has been reported to exist in three forms, the viable and culturable spiral form, the viable but non-culturable (VBNC) coccoid form, which are less virulent, and the non-viable degenerative *H. pylori* form[5]. It is their spiral shape that is thought to enhance their colonisation of the gastric mucosa. Whilst generally considered microaerophilic, there is now evidence that *H. pylori* can grow in humidified aerobic conditions[6].

The colonisation of *H. pylori* and its effect on resident gastric microbiota is relatively unknown. A study by Bik *et al*[7] assessed the human gastric microbiota from 23 gastric biopsy samples using 16S rDNA and subsequently found that the presence of *H. pylori* had no effect on the microbial profile of the gut[7]. A recent study investigated the effects of *H. pylori* on the gastric microbiota in a Rhesus macaque model. The authors found no significant impact upon the non-*Helicobacter* taxa after *H. pylori* challenge[8]. However, it appears that the microbial profile of the gut may have an effect on the degrees of pathogenicity of *H. pylori*. A germ-free gastric cancer mouse model showed less symptoms of disease and a later onset of neoplasia upon *H. pylori* infection when compared to those mice with a typical gastric microbiota profile[9].

As an avid coloniser of the gastric mucosa *H. pylori* must possess a number of characteristics that include flagella, adhesions, urease production, and biofilm forming ability[10,11]. The importance of the biofilm forming potential of *H. pylori* is fundamental to its pathogenicity. The formation of a biofilm is a virulence mechanism that aids in the enhancement and longevity of *H. pylori* in "unfriendly" and hostile environments, such as in the human stomach and the natural environment.

H. pylori was first found to demonstrate an ability to form *in vitro* biofilms in the early and late 1990s with solid evidence of this ability reported by Stark *et al*[12] in 1999. More recent reports on the ability of *H. pylori* to form biofilms within *in vitro*[13,14] and *in vivo* environments, specifically the gastric mucosa, have now been demonstrated[14-16]. In particular the *H. pylori* strain TK1402 isolated from a patient with duodenal and gastric ulcers has been shown to have very strong biofilm forming ability both inside and outside the host[14,15,17-20]. In this mode of growth it is likely that *H. pylori* is protected from external perturbations[18,21].

Biofilms can develop on both biotic and abiotic surfaces through the conversion of microorganisms in a free-floating or planktonic state, to a sessile state, where they become attached onto a surface. Once microorganisms attach onto a surface they proliferate, produce extracellular polymeric substance (EPS) and become firmly attached to that surface. The matrix of the biofilm is known to be composed of polysaccharides, extracellular DNA (eDNA), lipids and proteins that form the "house" of the biofilm[22,23]. It is the biofilm and the ability of microorganisms to form biofilms that form an essential element, aiding in their persistence, survivability, and recalcitrance to antimicrobial interventions and the host's immune response. Furthermore the ability of pathogenic microbes to survive within diverse and hostile environments is enhanced significantly when growing within a biofilm. Growth within a biofilm is known to cause and exacerbate infections and is responsible for prolonging infection, leading to chronicity[24].

A biofilm is dynamically and structurally complex and is often referred to as a "living organism" due to its ability to adapt to external perturbations. Of particular concern with biofilms of public health significance is the fact that sections of biofilms can easily detach or shear off, enabling these sections or individual bacteria to recolonise other surfaces. Detachment or dissemination from the biofilm can be achieved by the dispersal of single cells or the detachment/shedding of large cellular aggregates. Both situations constitute a concern to public health particularly where fluid resides, as microbial dissemination is enhanced *e.g.*, catheters, blood stream, drinking water[24]. Further to this there is growing evidence that within a biofilm the horizontal transfer of genes can occur, leading to large variations in *H. pylori* strains, particularly in one host, enhancing their survival and immune evasion. Moreover, gene transfer *in situ* has an important role to play in immunological effectiveness and eradication of pathogens by the host[25]. In addition to this it is well documented that when microorganisms are growing within a biofilm they have increased tolerance to antimicrobial agents[26].

It is the aim of this paper to review the ability of *H. pylori* to form biofilms *in vivo* and *in vitro* and to address the inherent mechanisms considered to significantly enhance its persistence within the host and in external environments. Furthermore, the dissemination of *H. pylori* in the external environment and within the human body and its impact upon infection control shall be discussed.

TRANSMISSION OF *H. PYLORI*

The routes of transmission of *H. pylori* are said to occur via an array of different pathways[27,28]. Although *H. pylori* are considered to be pathogens commonly associated with the human stomach, Brown proposed that *H. pylori* are able to survive in environments that are external to that of the human stomach[29]. Dental plaque has also been reported to contain *H. pylori;* however, in plaque, *H. pylori* are thought to only exist in a transient state[30-32]. Young *et al*[2] reported that both the spiral and viable coccoid form of *H. pylori* are present in the oral cavity. Souto and Colombo found *H. pylori* in a subgingival biofilm in 11% of periodontally healthy patients compared to 50% of patients suffering from periodontitis[33]. The

authors proposed that biofilm formation in the oral cavity should be considered as a potential reservoir for *H. pylori*.

There is growing evidence to suggest that *H. pylori* may reside in potable water systems[11]. In general, waterborne bacteria can adhere to surfaces by aggregating to form biofilms[26]. Information regarding the exact ecological niche where *H. pylori* reside and persist outside of the human host is limited. Despite this, there is growing evidence that external reservoirs of *H. pylori* may exist, potentially aiding transmission to the host. Furthermore there are also reports that *H. pylori* may have, as part of its life cycle, a zoonotic component. However, further scientific evidence of cultivability will be required to fully support this area.

The ability to form biofilms and the cell morphology and architecture formed depends greatly on the support material. To date however, in potable water supplies there is not enough substantial evidence that *H. pylori* within the viable state, plays a role in the development of a biofilm. Despite this, there is significant evidence that, in terms of epidemiological evidence, the risk of acquiring *H. pylori* increases in individuals who drink well water and river water or swim in pools, streams and rivers in particular[27,34-37]. Consequently, environmental water is considered a risk for the acquisition of *H. pylori* and therefore *H. pylori* biofilms in these environments should be a very important consideration when investigating reservoirs of source. The association of *H. pylori* with biofilms in water distribution systems can offer bacteria protection from disinfection and protozoan predation[38]. The challenge however, remains to determine the importance of waterborne *H. pylori*. It may be possible that a specifically adapted form of *H. pylori*, or simple *H. pylori* within a biofilm, may be required for persistence and transmission[39].

Although based on scientific logic, if *H. pylori* is able to survive and persist outside of the human host, its ability to develop a biofilm and survive within a biofilm may well help to answer fundamental questions regarding acquisition and potential eradication, particularly in the developing world.

THE DETECTION OF *H. PYLORI* IN THE ENVIRONMENT AND HOST

The ability of *H. pylori* to transform from a highly virulent, spiral shaped bacteria to a less virulent, non-culturable coccoid state, presents difficulties in the successful detection of this bacterium in both an environmental and clinical setting. In particular, the VBNC coccoid state is thought to arise under less favourable conditions, making the identification of *H. pylori* from water sources, particularly *H. pylori* within biofilms, unlikely using traditional culture methods.

Molecular methods such as real-time polymerase chain reaction (PCR) have been used to identify *H. pylori* in both spiral and coccoid states. Linke *et al*[40] used real-time PCR to target the ureA subunit of the *H. pylori* urea gene to identify *H. pylori* in drinking water biofilms. This *in vitro* study demonstrated successful identification of *H. pylori* from biofilms in silicone tubing, an imitation of drinking water systems. The study not only highlighted the capability of *H. pylori* to form biofilms in such a system but also emphasised the potential of using real-time PCR as a viable detection method. Although it is clear that more research into the identification of different strains of *H. pylori* using this method should be considered.

In terms of identification within the host, a very recent research paper by Fontenete *et al*[41] demonstrated the use of fluorescent *in situ* hybridisation (FISH) to identify *H. pylori* in culture and human gastric biopsies. This study mimicked *in vivo* conditions using gastric biopsies and modified the FISH method by replacing toxic chemicals, giving rise to the opportunity of using this method, given further development and trials, in *in vivo* situations.

THE ABILITY OF *H. PYLORI* TO FORM A BIOFILM

The ability of *H. pylori* to form a biofilm has been documented for over 15 years with biofilm growth heightened in environments which are composed of high carbon:nitrogen ratios[12,39]. The ability of *H. pylori* to develop biofilms has been reported in many *in vitro* studies[12-14,17,42,43]. The specific knowledge regarding the ability of *H. pylori* to form biofilms has been made possible by observations using microscopic techniques in particular scanning electron microscopy (SEM) specifically on glass but also on other materials[17].

Further to this, Yonezawa *et al*[18] reported that the *H. pylori* strain TK1402 (isolated from a Japanese patient with both duodenal and gastric ulcers) was able to form a biofilm but was dependent on the flagella, their ability to form cellular aggregates, and its ability to produce outer membrane vesicles. This ability to form biofilms has been shown to be modulated by quorum sensing molecules; in particular the luxS proteins have been identified in *H. pylori*[44].

Quorum sensing within H. pylori biofilms

Quorum sensing is an intercellular method of communication between microorganisms using chemical signalling. Quorum sensing molecules can be enzymes or peptides depending upon the signalling system. The accumulation of these signalling molecules leads to an interaction with cytoplasmic DNA-binding receptor proteins such as the *lux* protein family, whereby quorum sensing genes are modulated. Quorum sensing molecules however do not always bind to receptor proteins intracellularly; peptide molecule binding can occur on cell membranes whereby signal transduction leads to gene regulation[45].

In the case of *H. pylori*, this bacterium expresses a

homolog of the *luxS* gene, a gene responsible for the production of the quorum sensing molecule, autoinducer 2 (AI-2)[45]. The *H. pylori luxS* homolog has been implicated in bacterial attachment. Cole *et al*[13] revealed a two-fold increase in the biofilm formation of *H. pylori luxS* mutants when compared to the wild-type control. The authors concluded that in some strains of *H. pylori*, a mutation in quorum sensing signalling actually increases biofilm formation[13]. Later work by Rader *et al*[46] demonstrated defective motility in *luxS* mutants and highlighted the importance of quorum sensing AI-2 molecules as a regulator of flagella-associated genes in *H. pylori*. Further work by Rader *et al*[47] revealed that the release of AI-2 molecules acts as a chemorepellent for *H. pylori*. At this stage, the authors hypothesised that this action may cause *H. pylori* to break away from the majority of the bacterial population, avoiding niche competition and encouraging *H. pylori* dispersal. In the context of both external environments and within the clinical setting of the host, quorum sensing within a *H. pylori* biofilm may encourage dispersal, a mechanism that may induce the likelihood of transmission to and from an external environment and the host, and dissemination within the host.

H. PYLORI GROWTH WITHIN BIOFILMS: THE IMPORTANCE OF COCCOID FORMS AND RESUSCITATION

Understanding the growth of H. pylori

In vitro studies are an important starting point in the understanding of the dynamics of *H. pylori* growth within a biofilm. In light of this, the study of *H. pylori* in biofilms present challenges in the laboratory; nevertheless, the growth of *H. pylori* has been documented to behave differently in different growth conditions.

Bessa *et al*[48] assessed the growth of *H. pylori* in four types of liquid culture medium to assess the physiological behaviour and growth standardisation of *H. pylori*. *H. pylori* in free-living and biofilm modes of growth were assessed in Brucella broth, brain heart infusion broth and Ham's F-12 medium supplemented with 2% fetal calf serum and Ham's F-12 without serum. Free-living growth was monitored for 72 h in each medium and characterised for bacterial density, cultivability, viability and morphology. Biofilm formation in the same medium was evaluated for biomass production, colony forming unit (CFU) counts and microscopic visualisation. Afterward, using Ham's F-12, the effect of amoxicillin and clarithromycin at sub-minimum inhibitory concentrations (sub-MICs) was evaluated on *H. pylori* biofilm formation and *luxS* gene expression. Differences in free-living growth were observed between the culture medium supplemented with serum and Ham's F-12 without serum. Biofilm formation was significantly dependent on the growth media used. Ham's F-12 appeared to be a good medium to support both growth phenotypes of *H. pylori*. Moreover, sub-MICs of antibiotics increased the biofilm formation and affected the *luxS* gene expression[48]. Optimising the growth conditions of *H. pylori*, especially in the biofilm mode, will be helpful to perform more accurate in-depth studies that will increase the knowledge about *H. pylori* biofilms, which should be a target to eradicate resistant infection. Humidified conditions with 5%-7% oxygen and 7%-10% CO_2 with some H_2 or 10% CO_2 are also reported to be ideal for the growth of *H. pylori*[49]. However, the expression of catalase and superoxide dismutase (SOD), allows *H. pylori* to persist in higher levels of oxygen[50,51].

H. pylori biofilms, VBNC coccoid phenotypes and resuscitation

The emergence of VBNC pathogens has been of much interest in recent years due to the notion that this state is a form of survival and protection.

The VBNC coccoid form of *H. pylori* is formed during stress and starvation[52]; therefore it is in this form in which *H. pylori* is thought to reside in biofilms.

It has been reported that atmospheric conditions enhance the formation of VBNC coccoid *H. pylori* which has been suggested to resemble the same characteristics of persister cells documented in biofilms[11,53]. Furthermore, these cells then have the ability to resuscitate and lead to infection recurrence[54,55]. Cellini *et al*[20] identified the presence of *H. pylori* in gastric mucosa biopsies of patients treated for *H. pylori* infection. In this study, patients were identified as harbouring *H. pylori* through culture methods or, if non-culturable, the molecular method, reverse transcriptase polymerase chain reaction (RT-PCR). Scanning electron microscopy (SEM) of biopsies from patients with culturable samples, revealed prevalent spiral forms, co-existant with coccoid forms embedded within a matrix. In non-culturable cases, SEM showed the presence of coccoid clusters in a matrix that was shown to be biofilms, through the further identification of the *luxS* quorum sensing gene[20]. This study highlighted the importance of *H. pylori* biofilms, the presence of coccoid forms within the biofilm and resistance. Furthermore, it provided insight into the prevalence of coccoid forms in the gastric mucosa. With this is mind, it is important to focus research on the identification of these VBNC coccoid forms, and more importantly, understand the mechanisms behind recalcitrant coccoid states and how they can phenotypically shift into more virulent spiral forms.

The resuscitation of a pathogen in a VBNC state is of great clinical importance, given the extensive dormancy within the host for years before infection reoccurrence; thus the host is incorrectly diagnosed as infection-free. Therefore it is important to distinguish between viable and culturable pathogens and VBNC states in order understand the mechanism behind reactivation. Such detection methods can include Live/Dead assays and RT-PCR[40,56,57]. There have been several reported factors that induce resuscitation in a number of pathogenic spe-

cies of bacteria such as temperature shifts, peptidoglycan hydrolases and the release of human norepinephrine following tissue injury[58].

Earlier studies such as research by Cellini *et al*[59], stressed the importance of evaluating the survival potential of VBNC coccoid *H. pylori*. In this study, *H. pylori* ATCC 43504 was grown *in vitro* until a VBNC coccoid state was achieved, whereby "resuscitation" was then attempted using heat, pH and sonication shock methods. Unfortunately the authors were not confident in whether true resuscitation actually occurred, or whether it was simply a re-growth of undetected culturable cells. Richards *et al*[60] sought to create a modified resuscitation broth containing serum and lysed erythrocytes for *H. pylori* in the VBNC state. The resuscitation of *H. pylori* was recorded and the assessment of a gene involved in growth repression (*cdrA*) showed low expression in resuscitated *H. pylori*. These results show that although the *cdrA* gene is probably not responsible for loss of cultivability in *H. pylori*, the modified broth can be successfully used to resuscitate and therefore explore other possible mechanisms.

THE SURVIVAL AND PERSISTENCE OF *H. PYLORI* AND BIOFILMS

The ability to *H. pylori* to persist as a infectious entity and resist the armoury of antimicrobials employed to eradicate it, is considered to be due to both genetic variability but in addition, the ability of *H. pylori* to form biofilms which significantly aids its survival[15,16,18,61]. The formation of a biofilm by *H. pylori* has been shown to enable its protection from fluctuations in pH due to its ability to over produce EPS[12,62]. Siavoshi *et al*[6] set up a study to identify two mucoid strains of *H. pylori* and compare their growth under aerobic and microaerobic conditions with that of a control *H. pylori* strain. The authors found that the EPS produced by the two strains could serve as a physical barrier to reduce the oxygen diffusion and uptake of antibiotics into the bacterial cell. The EPS aimed to protect them against the increasing levels of oxygen, osmotic stress, acidic pH, host immune system, and antibiotics. The authors concluded that production of EPS by *H. pylori* could be an adaptation mechanism that facilitates bacterial survival and growth. This survival strategy would prevent bacterial removal by the host defence factors and antimicrobial therapy. Furthermore it would aid the persistent and long-term infection of *H. pylori* in the stomach and possibly the environment.

Survival and persistence in the environment

H. pylori in the viable and culturable form has been shown to survive > 10 d, whereas the VBNC coccoid form has been reported to survive for up to 1 year in fresh water[63]. Within distilled water West *et al*[64] reported that *H. pylori* can survive > 14 d, similar to that in saline, and > 7 d in sea water. More recent studies have shown that *H. pylori* can survive in deep ground water[65]. Interestingly numerous studies have reported that *H. pylori* are able to survive within a cultivable state for numerous weeks in water and other natural systems when compared to that of growth in nutrient rich conditions. The adaptation of *H. pylori* in different environments is reported to be intrinsic and consequently this may assist in the survival of the bacterium in the diverse environments outside of the human host. This potential persistence in the environment may not only be due to its ability to form biofilms but also its ability to survive within a community of other microorganisms within a polymicrobial ecosystem. This ability to survive hostile environments is made possible by a number of factors mentioned above but also by the ability of *H. pylori* to produce peptides[15].

An environment that has been reported to aid the survival of *H. pylori,* is that of water or more specifically in reference to public health, potable water - an oligotrophic environment that contrasts significantly to that of the gastric mucosa.

Mackay *et al*[66] and Park *et al*[67] first provided evidence that biofilms in water distribution systems may harbour *H. pylori*. Within these studies *H. pylori* was found incorporated into a laboratory-scale biofilm and persisted for over 8 d. Further to this Bunn *et al*[68] utilised 16S rDNA sequences and provided further evidence that *H. pylori* can survive in biofilms within water. Azevedo *et al*[21] and Bragança *et al*[69], have also shown that *H. pylori* may be present on pipe samples in drinking water systems which remain adhered and grow as biofilms. However, in this study it was found that a lack of recovery using culturable techniques occurred quickly over time indicating that *H. pylori* enters a non-culturable state in more "hostile" environments to that of the gastric mucosa. The survival of *H. pylori* in well water has also been documented, suggesting this is related to the ability of *H. pylori* to integrate into biofilms[69,70]. Potentially substratum material used in conjunction with both domestic and distribution systems are known to be one of the factors affecting the growth of biofilms. Subsequently, Azevedo *et al*[21] showed that *H. pylori* was able to adhere to different plumbing materials. Watson *et al*[57] also demonstrated a close link between *Helicobacter* DNA in showerhead biofilm used in domestic households.

All the research findings above support the concept that water may provide a route for the transmission of *H. pylori* outside of the human host.

Survival and persistence within the host

H. pylori has been detected and isolated from different regions of the human body. These have included gastric biopsies, gastric juice, dental plaque, saliva, bile and faecal matter, indicating its ability to colonise surfaces either transiently or in the case of the gastric mucosa, permanently[2,15,16,71]. The viable spiral-shaped *H. pylori* are more virulent and therefore infectious whereas the less virulent coccoid form have a reduced ability to colonise and induce inflammation and disease; an effect that has been

observed in animal models[5].

Biofilms are reported to serve as population-level virulence factors. Consequently this will enable the resident bacteria to acquire virulence attributes[25]. Biofilms provide ideal areas for bacterial horizontal gene transfer, which will help the production and provide a source of related strains, but with different antigenic and virulence profiles. Ultimately this will help to confuse the host immune system providing the bacterial community with a means to overwhelm the host's immune system[72].

Grande *et al*[73] reported that persistence of *H. pylori* might be associated with genetic variability and biofilm development. The researchers investigated the interaction between two clinical strains of *H. pylori* so they could understand the balance between strains that could co-exist in the same niche to be cooperative/competitive in their colonisation.

Interestingly *H. pylori* are a species that are genetically diverse. To date it has not been possible to isolate two identical DNA patterns from different hosts[74]. This of course is significant in evading the immune response from the host and consequently will favour the survival of *H. pylori*. Such a difference may explain the long-term colonisation that occurs in some hosts. There is a high level of genetic recombination within biofilms[75]. It is within the biofilm that horizontal gene transfer can occur as evident by high levels of eDNA detected in *H. pylori* biofilms[76]. As the biofilm is highly tolerant to the host's immune response, the availability of eDNA which is evident in the biofilm matrix could then be acquired by other *H. pylori*. This may therefore lead to the development of highly virulent strains of *H. pylori* in the host leading to their persistence.

ROLE OF BIOFILMS IN DISSEMINATION AND DISPERSAL OF *H. PYLORI* IN THE NATURAL ENVIRONMENT AND THE HOST

The dissemination of *H. pylori* is thought to occur through person-to-person contact but it is now also evident as demonstrated above, that *H. pylori* may also reside in drinking water systems. Whether in planktonic or biofilm form, albeit in the human stomach or external water supplies; the spread of this bacterium in such adverse environments is inevitable. With *in vitro* evidence of *H. pylori* residing in these environments in biofilm form, it is important to contemplate another method of dissemination. Not only do biofilms demonstrate increased resistance towards antimicrobials; biofilms possess another mechanism that greatly impacts upon transmission and dissemination within the host. "Dispersal" is a mechanism whereby members of the microbial community within a biofilm, detach and attach to new surfaces, effectively colonising a new site[77]. It is highly possible that dispersal has great impact on the dissemination of *H. pylori* not only within the host but also in the external environment, increasing the likelihood of transmission.

Dispersal can be described in three stages; the first being the detachment of bacterial cells from the biofilm, followed by the translocation of cells to a new site and finally the attachment of these cells to the new surface[77]. Given the adverse and hostile environment both outside and within the host, the dispersal of *H. pylori* may seem like an unavoidable process. However, in many microbial biofilms, dispersal is thought to be a carefully controlled mechanism. Bacterial cells that reach the end of their biofilm life cycle become differentiated and highly motile. These dispersal cells are specialised in that they are regulated by the intracellular molecule cyclic-di-GMP (c-di-GMP). In general, it is thought that a reduction in c-di-GMP leads to dispersal. Furthermore, genes that are associated with motility such as the flagellum are up-regulated[78].

In terms of *H. pylori* dispersal within biofilms, research to support this mechanism in both the environment and in the human body is lacking. Evidence that does indicate that this is a likely occurrence in *H. pylori* biofilms relate to that of *H. pylori* motility within biofilms.

It has been known for over a decade now that motility is essential for the survival and successful colonisation of *H. pylori* within the host[79].

As mentioned earlier, research by Rader *et al*[47] showed that the presence of AI-2 quorum sensing molecules that can be synthesised by *H. pylori*, act as a chemorepellent, affecting motility. Therefore the formation of *H. pylori* biofilms within the host and in the environment, whereby quorum sensing is likely to occur, may encourage the dispersal of cells from the biofilm and thus new sites of infection.

H. PYLORI ERADICATION

Environmental eradication of H. pylori

Early research by Baker *et al*[80] has shown that *H. pylori* demonstrates resistance to low dosages of free chlorine that ordinarily kill the coliforms such as *Escherichia coli*. Consequently areas in water distribution systems may not prevent the entry and potential proliferation of *H. pylori* in water. Further studies by Mazari-Hiriart *et al*[81] and Moreno *et al*[82] have demonstrated that drinking water treatments employed to date may be ineffective particularly when *H. pylori* are present in the coccoid shape, which is a well known VBNC and a potentially infective state of *H. pylori*.

Baker *et al*[80] and Johnson *et al*[83] demonstrated that *H. pylori* is inactivated by chlorine. However, their studies and conclusions did not recover culturable cells but reported only on the VBNC state. A more recent study by Moreno *et al*[82] also demonstrated the survival of *H. pylori* but again only in the VBNC state. Unfortunately all these studies did not take into account the survival and association of *H. pylori* in biofilms and the toler-

ance when grown as part of a biofilm[84]. A later study by Gião *et al*[85] demonstrated that viable *H. pylori* can survive in the viable state in biofilms. The efficacy of chlorine treatment on a biofilm that contained this bacterium was investigated further. In later studies, Gião *et al*[85] found that using a specific peptide nucleic acid (PNA) probe it could be demonstrated that *H. pylori* persist inside biofilms that had been exposed to chlorine at 0.2 and 1.2 mg/L. This occurred for at least 26 d. In this study, no culturable cells were recovered. However when viability stains were employed *H. pylori* was observed suggesting that it could survive within a biofilm at this concentration of chlorine[86].

If *H. pylori* are disseminated into the water cycle and enter water distribution systems, it is possible that routinely used water treatment methods and disinfectants presently employed may not be as effective as once thought. This seems to be due to the ability of *H. pylori* to survive within a biofilm.

Eradication within the host

The first-line therapy for the eradication of *H. pylori* involves the combination of a proton pump inhibitor in conjunction with either clarithromycin (CLR) or metronidazole, and amoxicillin[87-89]. The antibiotic CLR is a macrolide antibiotic that is known to bind to the 50S subunit of the bacterial ribosome and thereby inhibiting the translation of peptides, leading to the inhibition of growth. However, of growing significance to *H. pylori* eradication is the increasing problem of CLR-resistance[88-92].

H. PYLORI RESISTANCE WITHIN BIOFILMS

There are growing reports regarding the resistance of *H. pylori* to clarithromycin, the common antibiotic which is used in its eradication in the human host[88]. The occurrence of CLR resistant *H. pylori* is very common with ranges being reported between 10% to 30%[93,94]. The basis of resistance is a point mutation in the domain V loop of the 23S rRNA gene (commonly an adenine-to-guanine transition at position 2142 or 2143)[88,90-96].

Furthermore Yonezawa *et al*[97] investigated the effects of *H. pylori* biofilm formation *in vitro* on CLR susceptibility. Within this study CLR susceptibility of intermediate (2-d) and mature (3-d) *H. pylori* biofilms on glass coverslips was determined. Concentrations of CLR applied to the biofilm ranged from 0.03 to 0.5 mg/mL. It was found that the biomass of the *H. pylori* biofilm increased after treatment with CLR at minimum inhibitory concentration levels by up to 4-fold (2-d biofilm) and 16-fold (3-d biofilm). In addition to this the minimum bactericidal concentrations of CLR against cells in a biofilm was higher (1.0 mg/mL) for the biofilm-grown cells when compared with the planktonic cells (0.25 mg/mL). Furthermore the expression of efflux pump genes significantly increased in the biofilm cells. Overall, this study demonstrated that *H. pylori* biofilm formation decreases the susceptibility to CLR. In addition it was found that *H. pylori* CLR resistant mutations were generated more frequently in biofilms than in planktonic cells. *H. pylori* has numerous constitutive genes which may help to rapidly neutralise oxidative antimicrobials. The rapid expression of constitutive enzymes may help to assist the survival of *H. pylori* in the environment. A survival strategy is the formation of coccoid phenotypes.

CONCLUSION

The ability to grow and proliferate within a biofilm is significant to the longevity, survival and also dissemination of *H. pylori*. Growth within a biofilm is a significant risk factor in both its eradication and treatment and therefore its persistence both within the host and the environment. Within this state, its recalcitrance is enhanced and its ability to acquire genes enhancing virulence is evident. This adaptation is effective for its survival, genetic variability and persistence. The characteristics of *H. pylori* provide evidence of survival in the environment and therefore acquisition is heightened. It is well known that *H. pylori* in stressful environments convert from the virulent infectious spiral phenotype to that of the less virulent VBNC coccoid state. It is within this VBNC coccoid state that *H. pylori* is thought to reside within biofilms. Biofilms have been associated with persistent infections and increased resistance to antimicrobial action. Thus, the ability of *H. pylori* to resuscitate and revert from the coccoid to spiral form is a mechanism that requires attention in terms understanding the factors that may lead to infection reoccurrence both in the host and the external environment.

The dissemination of *H. pylori* is significant in its acquisition by the host. Person-to-person transmission is a strong risk factor. However, there is more evidence growing following an initial report in early 2000, that contaminated water may be an important conduit for dissemination and acquisition. However the lack of evidence relating to the presence of *H. pylori*, particularly in biofilm form, in the environment is apparent and may be due to the transformation of *H. pylori* from cuturable spiral form to the VBNC coccoid form. The detection methods used to identify *H. pylori*, particularly in the VBNC coccoid state, need to be refined if successful identification of this microorganism is to be made.

The biofilm-forming potential of *H. pylori* means that eradication both within the host and the environment, is significantly reduced, which justifies the need to refine and develop treatment regimes and strategies that are more appropriate and effective than traditional therapies that have high failures rates in eradicating *H. pylori*. In the environment, present evidence suggests that traditionally used disinfectants are effective on planktonic *H. pylori* but little evidence exists on the effectiveness of antimicrobials on *H. pylori* in environmental biofilms. This environment, be it potable water biofilms or biofilms in

hot water systems in domestic houses, may be a possible reservoir for *H. pylori* and aid in its transmission and dissemination.

Appropriate anti-biofilm agents are therefore required to ensure that in the host, *H. pylori* can be eradicated fully and continuing dissemination does not occur.

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TOPIC HIGHLIGHT

WJGP 5th Anniversary Special Issues (1): *Helicobacter pylori*

Role of Toll-like receptors in *Helicobacter pylori* infection and immunity

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Abstract

The gram-negative bacterium *Helicobacter pylori* (*H. pylori*) infects the stomachs of approximately half of the world's population. Although infection induces an immune response that contributes to chronic gastric inflammation, the response is not sufficient to eliminate the bacterium. *H. pylori* infection causes peptic ulcers, gastric cancer and mucosa-associated lymphoid tissue lymphoma. Disease outcome is linked to the severity of the host inflammatory response. Gastric epithelial cells represent the first line of innate immune defence against *H. pylori*, and respond to infection by initiating numerous cell signalling cascades, resulting in cytokine induction and the subsequent recruitment of inflammatory cells to the gastric mucosa. Pathogen recognition receptors of the Toll-like receptor (TLR) family mediate many of these cell signalling events. This review discusses recent findings on the role of various TLRs in the recognition of *H. pylori* in distinct cell types, describes the TLRs responsible for the recognition of individual *H. pylori* components and outlines the influence of innate immune activation on the subsequent development of the adaptive immune response. The mechanistic identification of host mediators of *H. pylori*-induced pathogenesis has the potential to reveal drug targets and opportunities for therapeutic intervention or prevention of *H. pylori*-associated disease by means of vaccines or immunomodulatory therapy.

Key words: *Helicobacter pylori*; Toll-like receptor; Gastric epithelium; Monocyte; Macrophage; Dendritic cell; Cytokine; Lipopolysaccharide

Core tip: Eradication rates for *Helicobacter pylori* (*H. pylori*) infection have fallen. The development of therapeutic alternatives to antibiotics, such as immunomodulatory therapy and vaccines requires a clearer understanding of host-pathogen interactions. As Toll-like receptors are intimately involved in the regulation of inflammation in response to *H. pylori* and represent key activators of adaptive immunity, they represent a target for therapeutic manipulation. Elucidating innate immune signals triggered by *H. pylori* will provide an understanding of how the balance between pro-inflammatory and anti-inflammatory signals fine-tunes the response to infection and insight into how the immune response may be manipulated therapeutically to successfully eradicate the bacterium.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a gram-negative microaerophilic flagellated bacterium that specifically infects

the stomachs of approximately 50% of the world's population. Infection is thought to be acquired in early childhood and persists for life if left untreated, despite triggering vigorous innate and adaptive immune responses[1-4]. Prevalence of *H. pylori* infection varies throughout the world and is associated with lower socioeconomic conditions[5]. Most infected individuals are asymptomatic. However, infection may cause chronic gastritis and confers a 1%-10% risk of developing gastric or duodenal ulcers, a 0.1%-3% risk of developing gastric adenocarcinoma, and < 0.01% of developing mucosa-associated lymphoid tissue (MALT) lymphoma[2]. Disease risk varies in different populations and is associated with host genotype, strain-specific bacterial components and environmental factors. *H. pylori* colonization of the gastric mucosa is followed by infiltration of polymorphonuclear leukocytes, monocytes and lymphocytes[6]. Mucosal levels of pro-inflammatory cytokines and chemokines such as interleukin-6 (IL-6), interleukin-8 (IL-8), tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β) are significantly higher in *H. pylori*-positive compared to *H. pylori*-negative gastric specimens[7-9]. Although, *H. pylori* infection induces an immune response that contributes to chronic gastric inflammation, the response is not sufficient to eliminate the bacterium[6,10]. Progression of disease from superficial gastritis to gastric cancer is linked to the severity of the host inflammatory response[11-13].

All consensus guidelines recommend eradication of *H. pylori* in symptomatic individuals using a standard first-line triple therapy consisting of a proton pump inhibitor together with the antibiotics clarithromycin and amoxicillin or metronidazole[4]. However, eradication rates have fallen in recent years in line with a rapid increase in antimicrobial resistance[14]. The most recent multicentre European assessment on *H. pylori* antimicrobial susceptibility has indicated that resistance rates for metronidazole and clarithromycin are 34.9% and 17.5% respectively[15]. Clarithromycin resistance has almost doubled in Europe in the last 10 years[15]. Furthermore, a high resistance rate of 14.1% has emerged for levofloxacin, which is used in rescue therapy for *H. pylori* infection[15]. This rapid emergence of antibiotic resistant strains of *H. pylori* is a cause for concern. The development of therapeutic alternatives to antibiotics, such as immunomodulatory therapy and vaccines requires a more lucid understanding of host-pathogen interactions. The mechanistic identification of host mediators of *H. pylori*-induced pathogenesis has the potential to reveal drug targets and opportunities for therapeutic intervention or prevention of *H. pylori*-associated disease.

The immune system consists of innate and adaptive immunity, that cooperate to efficiently control infections. The evolutionary conserved innate immune system provides the first line of defence against invading microbes, whereas the adaptive immune system is developed in later phases of infection and is highly specific, long lasting and possesses immunological memory[16]. Innate immune recognition of microbes is mediated by families of pathogen recognition receptors (PRRs), which recognize pathogen-associated molecular patterns (PAMPs) that are broadly shared by pathogens[17]. Upon PAMP recognition by a particular PRR, cell signalling cascades are triggered that are necessary for initiation of the host response. Additionally, PRR signalling induces the maturation of the major antigen presenting cells, dendritic cells (DCs), and the subsequent induction of adaptive immunity[17].

Gastric epithelial cells of the stomach mucosa represent the first line of innate immune defence against *H. pylori*, and respond to infection by initiating numerous cell signalling cascades[11]. PRRs of the Toll-like receptor (TLR) family have been shown to mediate many of these cell signalling events. In particular, a key role for TLR2 has been described in the response to *Helicobacter* in multiple cell contexts[11,18-21]. Recent data also suggest associations between TLR2 polymorphisms and the severity of intestinal metaplasia in *H. pylori*-positive patients[22] and with gastric cancer risk[23]. Additionally, polymorphisms in the TLR1 gene, which encodes a TLR2 co-receptor, are associated with *H. pylori* prevalence[24].

TLRS AND PATHOGEN RECOGNITION

TLRs are the most widely studied of the PRRs. Members of the TLR family are type I transmembrane proteins, consisting of a leucine-rich repeat-containing ectodomain involved in PAMP recognition, a transmembrane region and an intracellular portion that harbours a Toll-IL-1 receptor (TIR) domain involved in the activation of downstream signalling pathways. There are 10 TLR genes in humans[25]. TLRs are expressed on the cell surface or associated with intracellular vesicles, such as endosomes[16,17] (Figure 1). TLR1, TLR2, TLR4, TLR5 and TLR6 bind their respective ligands on the cell surface and recognize microbial membrane components such as lipids, lipoproteins and proteins[16,17]. TLR3, TLR7, TLR8, TLR9 are found in intracellular vesicles such as the endosome or lysosome and the endoplasmic reticulum, and are mainly involved in the recognition of microbial nucleic acids[16,17].

TLR4 was the first human TLR to be identified and recognizes bacterial lipopolysaccharide (LPS), which is a major constituent of the outer membrane of gram-negative bacteria[26]. LPS is a surface exposed glycolipid that consists of a hydrophobic membrane anchor portion, known as lipid A, and a non-repeating core oligosaccharide coupled to a distal polysaccharide (O-antigen) that extends from the bacterial surface[27,28]. The lipid A domain is responsible for the endotoxic properties associated with LPS. There is considerable LPS structural variability, due to diversity in both the chemical composition of the polysaccharide O-antigen and in lipid A variations, which contribute to the ability of some gram-negative bacteria to evade immune detection[27,28]. Smooth LPS is composed of a polysaccharide O-antigen side chain and has complete core oligosaccharides,

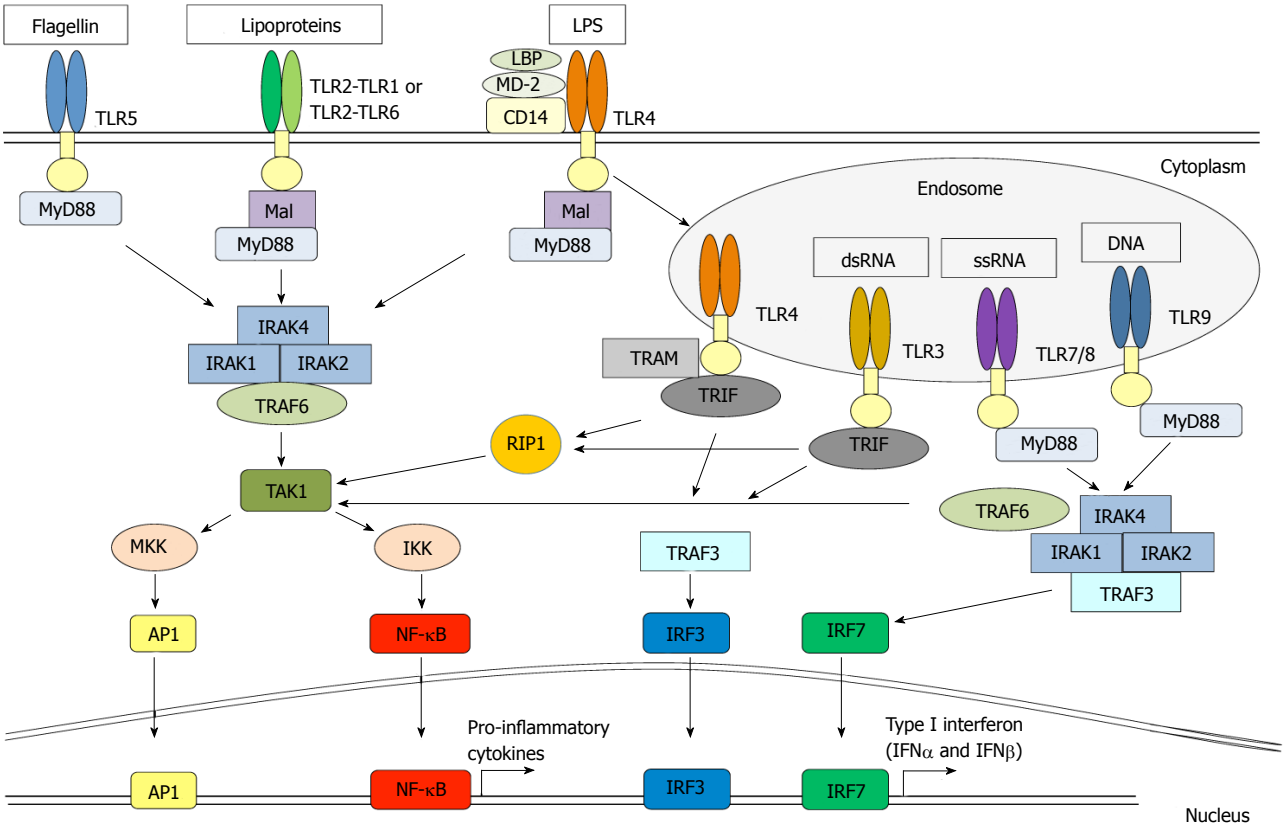

Figure 1 Toll-like receptor signalling. Toll-like receptors (TLRs) are type I transmembrane proteins, consisting of a leucine-rich repeat-containing ectodomain involved in pathogen-associated molecular pattern (PAMP) recognition, a transmembrane region and an intracellular portion that harbours a Toll-IL-1 receptor (TIR) domain involved in adapter protein recruitment and the activation of downstream signalling pathways. TLR1, TLR2, TLR4, TLR5 and TLR6 bind to their ligands on the cell surface and recognize microbial membrane components. TLR3, TLR7, TLR8, TLR9 are found in intracellular vesicles and are mainly involved in the recognition of microbial nucleic acids. TLR signalling is initiated by ligand-induced receptor dimerization and TIR engagement with the adapter proteins MyD88 or TRIF. TLR4 localises from the cell membrane to endosomes to change signalling through MyD88 to TRIF. MyD88 is a central TLR adapter protein utilized by all TLRs, with the exception of TLR3, and transmits signals that result in the induction of inflammatory cytokines. The association between a TLR and MyD88 recruits members of the IRAK family. IRAK1 and IRAK4 are sequentially phosphorylated and dissociated from MyD88. This results in the activation of TRAF6, which in turn activates TAK1. TAK1 activates the IKK complex. In most resting cells, NF-κB is bound to the inhibitory IκB proteins (IκBα and IκBβ) in the cytoplasm. Upon activation of the IKK complex, IκB becomes phosphorylated and degraded, thus releasing NF-κB for translocation to the nucleus, where it interacts with promoters harboring κB binding elements. In addition, TAK1 stimulation results in the induction of MAP kinases kinases (MKKs) that activate p38, JNK and ERK, resulting in the subsequent activation of AP-1. In the case of TLR4, and to a lesser extent TLR2, the activation of this pathway involves the bridging adapter protein MAL, which links MyD88 to the TLR. The adapter protein TRIF is involved in the MyD88-independent TLR4 pathway, as well as the TLR3 signalling pathway. TRAM links TRIF to TLR4. Endosomal TLR-mediated signalling leads to the induction of type I interferon through the activation of the transcription factors IRF3 and IRF7.

whereas rough LPS lacks O-antigen and has shorter core oligosaccharides[16]. MD-2 is closely associated with TLR4 on the cell surface and is required for strong inflammatory cytokine induction in response to LPS. LPS-binding protein (LBP) and CD14 are also involved in the TLR4-mediated response to LPS[16]. Cells lacking CD14 are not responsive to smooth LPS but still respond to rough LPS or lipid A[16].

TLR2 recognizes a number of PAMPs on a variety of microorganisms, including zymosan from fungi, triacyl lipopeptides from bacteria and mycobacteria, diacyl lipopeptides from mycoplasma, and peptidoglycan and lipoteichoic acid from gram-positive bacteria[16,17]. TLR2 distinguishes between PRRs by hetero-dimerization with TLR1, TLR6, dectin-1 or CD14. TLR2 heterodimerizes with TLR1 to recognize triacylated lipopeptides from gram-positive bacteria[29,30] or with TLR6 to recognize diacylated lipopeptides, lipoteichoic acid and zymosan[31,32]. CD14 is involved in the recognition of diacylated lipopeptide, whereas the C-type lectin receptor dectin-1 collaborates with TLR2 in the recognition of β-glucan[16] found in the cell walls of fungi and yeasts. TLR2 has also been shown to recognize atypical forms of LPS[33-37]. TLR5 recognizes flagellin[38], a protein component of bacterial flagella. A role for TLR10 has not yet been shown, but the TLR10 sequence is most similar to TLR1 so TLR10 may heterodimerize with TLR2[25]. TLR3 recognizes double stranded RNA[39], which is a major component of many viruses. TLR9 is the receptor for CpG-rich hypomethylated DNA motifs[40], frequently found in bacterial DNA. TLR9 also responds to herpes virus DNA[41]. TLR7 and TLR8 sense single-stranded viral RNA[42-44].

TLR SIGNALLING

Upon PAMP recognition, TLRs trigger cell signalling pathways resulting in (1) the activation of the transcription factors nuclear factor-κB (NF-κB), activating protein-1 (AP-1) and interferon regulatory factors (IRFs); (2) expression of inflammatory cytokines, antimicrobial peptides and type I interferon (IFN); and (3) the subsequent recruitment of neutrophils, activation of macrophages and dendritic cells and the induction of IFN-stimulated genes. The specific response triggered by an individual TLR depends on the recruitment of a single or combination of TIR-domain containing adapter proteins[17]. MyD88 (myeloid differentiation primary response protein 88) is a key TLR adapter protein utilized by all TLRs, with the exception of TLR3, and transmits signals that result in the induction of inflammatory cytokines (Figure 1). The association between a TLR and MyD88 recruits members of the interleukin-1 receptor-associated kinase (IRAK) family. IRAK1 and IRAK4 are sequentially phosphorylated and dissociated from MyD88. This results in the activation of tumor necrosis factor receptor-associated factor 6 (TRAF6), which in turn activates transforming growth factor B-activated protein kinase 1 (TAK1). TAK1 activates the IKK [inhibitor of NF-κB (IκB) kinase] complex. In most resting cells, NF-κB is bound to the inhibitory IκB proteins (IκBα and IκBβ) in the cytoplasm. Upon activation of the IKK complex, IκB becomes phosphorylated and degraded, thus releasing NF-κB for translocation to the nucleus, where it interacts with promoters harboring κB binding elements to regulate gene transcription[45]. In addition, TAK1 stimulation results in activation of MAP kinase kinases (MKK) leading to the induction of the MAP kinases p38, JNK and ERK, resulting in the subsequent activation of AP-1[45] (Figure 1). In the case of TLR4, and to a lesser extent TLR2, the activation of this pathway involves the bridging adapter protein, MAL (MyD88 adapter-like, also known as TIR-domain-containing adapter protein, TIRAP)[46-49] , which links MyD88 to the TLR. The TIR-domain-containing adapter protein inducing IFNβ (TRIF, also known as TICAM1) is involved in the MyD88-independent TLR4 pathway, as well as the TLR3 signalling pathway[50-53] (Figure 1). TRIF-related adapter molecule (TRAM, also known as TICAM2) links TRIF to TLR4[54-56]. Endosomal TLR-mediated signalling leads to the induction of type I interferon through the activation of the transcription factors IRF3 and IRF7[25] (Figure 1).

THE ROLE OF TLRS IN *H. PYLORI* INFECTION

Epithelial cells

As gastric epithelial cells represent the first point of contact between *H. pylori* and the host, there has been a focus on the individual TLRs involved in the response to *H. pylori* infection in this cell context (Table 1). Expression of numerous TLRs has been confirmed in many gastric epithelial cells lines, including AGS, MKN28, MKN45, NUGC3 and KATOIII[19,57-60]. In addition TLR2 has been detected in epithelial cells from human gastric biopsy samples, with increased TLR2 expression reported in samples from *H. pylori*-infected patients[61,62]. Increased TLR4 expression has also been reported in the gastric mucosa of *H. pylori*-infected patients[58]. The Goldberg laboratory have reported a role for both TLR2 and TLR5 during *H. pylori* infection of MKN45 cells; inhibition of TLR2 or TLR5 (but not TLR4) function using dominant-negative mutant constructs decreased *H. pylori*-driven NF-κB activation[19]. In order to assess the contribution of individual TLRs during *H. pylori* infection, many investigators have utilised human embryonic kidney 293 (HEK293) cells stably expressing specific TLRs. HEK293 cells act as a suitable negative control as they do not express TLR2 or TLR4 endogenously[19,63,64]. Indeed additional studies from the Goldberg group have supported a role for TLRs during *H. pylori* infection by demonstrating that over-expression of TLR2 or TLR5 in HEK293 cells enhanced NF-κB activation and IL-8, macrophage inflammatory protein 3α (MIP-3α) and growth regulated protein α (GROα) mRNA expression in response to *H. pylori*[19]. Others have also confirmed that TLR2 expression in HEK293 cells results in enhanced IL-8 expression following *Helicobacter* infection[7,11]. Using HEK-TLR2 cells, the Goldberg group subsequently utilised microarray analysis to identify 28 TLR2-dependent genes whose expression was altered in response to *H. pylori* infection[20]. A number of these genes demonstrated distinct expression patterns between AGS cells (which do not express TLR2 endogenously[20,62]) and MKN45 cells (which express TLR2[19,57])[20].

Monocytes/macrophages

Following *H. pylori* infection, epithelial cells release a variety of cytokines and chemokines leading to the recruitment of monocytes/macrophages to the gastric mucosa. Mononuclear cell infiltration in the lamina propria is characteristic of *H. pylori*-induced chronic infection[65]. Human monocytes and macrophages express a wide repertoire of PRRs. *H. pylori* has been shown to induce secretion of inflammatory cytokines (IL-1β, IL-6, IL-8) from peripheral blood mononuclear cells and IL-8 from purified human monocytes and monocyte-derived macrophages[11]. Different studies have implicated alternative TLRs in the *H. pylori*-mediated response in monocytes/macrophages (Table 1). Maeda *et al*[57] (2001) demonstrated that peritoneal macrophages from C3H/HeJ mice carrying a point mutation in the TLR4 gene showed decreased NF-κB activation and TNFα secretion compared with C3H/HeN macrophages in response to *H. pylori* infection. On the other hand, Gobert *et al*[66] (2004) found no significant difference in terms of IL-6 mRNA induction between peritoneal macrophages isolated from wild-type mice, TLR2-, TLR4- and MyD88-deficient mice in response to *H. pylori* infection. Using bone-

Table 1 Toll-like receptor involvement in the response to *Helicobacter pylori* infection in different cell types

Cell type	Cell type	TLR involvement	Readout of TLR activation	Ref.
Epithelial	MKN45	TLR2	NF-κB-dependent reporter gene activity	Smith *et al*[19]
	HEK-TLR2	TLR5		
	HEK-TLR5			
	HEK-TLR2	TLR2	MIP-3α mRNA expression	Smith *et al*[19]
			IL-8 and	
			GROα mRNA expression	
	HEK-TLR2	TLR2	IL-8 production	Mandell *et al*[11]
	HEK-TLR2	TLR2	mRNA expression of multiple genes	Ding *et al*[20]
	AGS			
	MKN45			
	HEK-TLR2	TLR2	IL-8 mRNA expression	Smith *et al*[7]
Monocytes and macrophages	Mouse peritoneal macrophages	TLR4	NF-κB activation by electro mobility shift assay	Maeda *et al*[57]
			TNFα production	
	Mouse peritoneal macrophages	TLR2-, TLR4- and MyD88-independent	IL-6 mRNA expression	Gobert *et al*[66]
	Mouse BMDMs	TLR2	IL-6 production	Mandell *et al*[11]
	Mouse BMDMs	TLR2	IL-6 and IL-1β production	Obonyo *et al*[67]
		TLR4	IL-10 and IL-12 production	
	Mouse BMDMs	MyD88	IL-6 and IL-12 production	Rad *et al*[69]
Dendritic cells	Mouse BMDCs	MyD88	MHC II and co-stimulatory molecule induction	Rad *et al*[69]
			IL-6, IL-12 and TNFα production	
			mRNA expression of multiple genes	
	Mouse BMDCs	TLR2	mRNA expression of multiple genes	Rad *et al*[18]
		TLR4		
		TLR9	IL-6 and IL-12 production	
	Mouse BMDCs	TLR2	IL-1β production	Kim *et al*[70]
	Mouse BMDCs	TLR2	IL-12, TNFα, IL-6 and IL-23 production	Sun *et al*[73]
B cells	Mouse B cells	MyD88	IL-6 and IL-12 production	Rad *et al*[69]
	Mouse B cells	MyD88	IL-10, IL-6 and TNFα production	Sayi *et al*[21]
		TLR2	CD80 and CD86 expression	
			Secretion of antibodies	

TLR: Toll-like receptor.

marrow derived macrophages (BMDMs) from knockout mice, Mandell *et al*[11] (2004) reported that the cytokine (IL-6) response to *H. pylori* was mediated by TLR2. *H. pylori*-infected BMDMs from wild-type or TLR4-deficient mice produced a robust cytokine response, whereas macrophages form TLR2-deficient mice were unresponsive. It is possible that alternative TLRs are involved in the *H. pylori*-mediated induction of individual cytokines within a particular cell context. Indeed, Obonyo *et al*[67] (2007) demonstrated that *H. pylori* induced IL-12 and IL-10 through TLR4/MyD88 signalling and IL-6 and IL-1β through TLR2/MyD88 signalling using BMDMs from knockout mice. As such, this study would suggest that *H. pylori* infection activates both TLR2 and TLR4 signalling in BMDMs leading to the secretion of distinct cytokines. This hypothesis is possible, given that individual *H. pylori* components have been suggested to trigger TLR2 or TLR4 signalling (Table 2).

Dendritic cells

In recent years, there has been an increasing interest in the mechanisms by which *H. pylori* initiates adaptive immunity and instructs the phenotype of the T cell response. During the activation of adaptive immunity, different T-helper (Th) cell subsets arise that exhibit characteristic patterns of cytokine secretion. As the major antigen presenting cells, DCs play a key role in the induction of the adaptive immune response. DCs express a wide range of PRRs[68] and possess the unique ability to capture antigen from the periphery and activate naïve T cells to direct T cell differentiation by producing three types of signals; antigen presentation, co-stimulation and cytokine secretion[18,69]. Rad *et al*[69] have shown that *H. pylori* activates DCs in a MyD88-dependent manner (Table 1). Production of pro-inflammatory cytokines (IL-6, IL-12 and TNFα), and induction of major histocompatibility complex class Ⅱ (MHC Ⅱ) and co-stimulatory molecules in MyD88-deficient DCs was impaired compared to wild-type cells following *H. pylori* stimulation. Further analysis of the *H. pylori*-controlled DC transcriptome by microarray analysis indicated that MyD88 was involved in the regulation of numerous genes involved in DC maturation, antigen uptake and presentation, as well as effector cell recruitment and activation[69]. *H. pylori*-mediated cytokine stimulation was also impaired in B cells and macrophages from the MyD88-deficient mice (Table 1). The *in vitro* findings were reflected *in vivo* in the form of reduced gastric inflammation and increased bacterial colonization following 4 mo *H. pylori* infection in MyD88-deficient mice, suggesting that the impaired immune response in MyD88-deficient mice enables better bacterial survival[69]. *Helicobacter*-specific

IgG2c/IgG1 ratios were reduced in MyD88-deficient mice, implying the involvement of the MyD88 pathway in the instruction of a Th1 phenotype[69]. Subsequent research by Rad *et al*[18] (2009) further characterized TLR-mediated signalling in DCs during *H. pylori* infection. They identified a MyD88-dependent component of the DC activation program that was induced by TLR2 and to a minor extent TLR4. Microarray analysis of *H. pylori*-stimulated DCs showed complementary, redundant and synergistic interactions between TLRs. Using TLR2-deficient cells the anti-inflammatory cytokine IL-10 was identified as a TLR2-dependent *H. pylori* responsive gene in DCs[18]. In addition, they demonstrated that IL-6 and IL-12 production was inhibited by approximately 50% in TLR2/TLR4/TLR9-deficient BMDCs compared to TLR2/TLR4-deficient cells in response to *H. pylori* infection, implying that TLR9-dependent recognition of *H. pylori* in DCs contributes to the cytokine response[18]. More recently Kim *et al*[70] (2013) have also implicated TLR signalling in response to *H. pylori* by demonstrating a role for TLR2 in *H. pylori*-induced IL-1β production in mouse BMDCs.

Although *H. pylori*-infected individuals generate a strong immune response, they fail to eradicate the bacterium. Emerging evidence suggests that failure to eliminate *H. pylori* may be due to its ability to induce a regulatory T cell (Treg) response, as expression of the Treg marker Foxp3 is increased in *H. pylori*-infected gastric tissue compared to that of uninfected individuals[71,72]. Sun *et al*[73] (2013) have recently investigated the functional role of TLR2 signalling in BMDCs in response to *H. pylori* and the subsequent effects on T cell responses. Firstly, they demonstrated that *H. pylori*-infected BMDCs from TLR2-deficient mice exhibited impaired production of the pro-inflammatory cytokines that promote both Th1 responses (IL-12 and TNFα) and Th17 responses (IL-6 and IL-23) compared to wild-type cells. Additionally, this report suggests that *H. pylori* may skew differentiation of naïve T cells towards Th17 and Treg responses as opposed to Th1 responses, as *H. pylori*-stimulated BMDCs from TLR2 knock-out mice induced a higher splenocyte production of IFNγ (Th1 response) and lower production of IL-17 (Th17 response) and IL-10 (Treg response)[73]. *In vivo* analyses following *H. pylori* infection for 2 mo showed a lower degree of gastric *H. pylori* colonization in TLR2 knock-out mice and more severe gastritis, implying that the TLR2-mediated response to *H. pylori* promotes a bacterial survival advantage. Sun *et al*[73] also demonstrated that the gastric mucosa of the infected TLR2 knock-out mice had lower Foxp3, IL-10 and IL-17A expression, but higher expression of IFNγ compared to wild-type mice. The *H. pylori*-specific Th1 response was higher and the Treg and Th17 responses were lower in the spleens of infected TLR2 knock-out mice, suggesting that *H. pylori* mediates immune tolerance through TLR2-derived signals and inhibits Th1 immunity, thus evading the host defence[73]. It is noteworthy that the Rad *et al*[69] (2007) study suggested MyD88-dependent TLR signalling promotes a Th1 response in *H. pylori*-infected mice and is protective against *H. pylori* colonization, while the Sun *et al*[73] (2013) study implies that TLR2 signalling inhibits Th1 immunity, supports a Treg/Th17 response and promotes *H. pylori* colonization. It is possible that different TLR ligands from the same pathogen induce distinct but opposing signals. Furthermore, it is likely that the complementary, redundant and synergistic interactions between TLRs in DCs subsequently reported by Rad *et al*[18] (2009) contribute to the observations from studies involving MyD88-deficient mice.

B cells

Evidence suggests that B cells contribute to the immunepathogenesis of *H. pylori* infection[74]. Sayi *et al*[21] (2011) have demonstrated that B cells play a role in regulating T cell responses and gastric immunopathology in response to *Helicobacter*. Building on the finding by Rad *et al*[69] (2007) that TLR2 is required for *Helicobacter*- mediated IL-6 and IL-12 induction in B cells, Sayi *et al*[21] showed that cytokine production (IL-10, IL-6 and TNFα), surface expression of the activation markers CD80 and CD86 and induction of antibody secretion was impaired in *Helicobacter*-stimulated B cells from both MyD88- and TLR2-deficient mice compared to wild type control cells (Table 1). The *Helicobacter*-stimulated B cells induced IL-10-producing $CD4^+CD25^+$ T regulatory-1 (Tr-1)-like cells in a TLR2- and MyD88-dependent manner[21]. The Tr-1 cells acquired suppressive activity *in vitro* and suppressed excessive gastric *Helicobacter*-associated immunopathology *in vivo*, suggesting that TLR2-mediated signalling in B cells plays a role in regulating the balance of *Helicobacter*-specific T cell responses to prevent excessive Th1-driven immunopathology and promote mucosal homeostasis, but enabling bacterial persistence[21].

RECOGNITION OF DISTINCT *H. PYLORI* COMPONENTS BY SPECIFIC TLRS

LPS

Based on the involvement of TLRs in regulating immunopathology in the context of *H. pylori* infection, many investigators have set out to elucidate the contribution of individual *H. pylori* components to the control of TLR-driven innate immune responses. *H. pylori* LPS has a lower endotoxicity than other gram-negative bacteria such as *Escherichia coli* or *Salmonella enterica*[75-78]. Although there has been substantial investigation into the innate immune response to *H. pylori* LPS, there have been conflicting findings with regard to the TLR responsible for its recognition (Table 2). Some studies have implicated the classic gram-negative bacterial LPS receptor TLR4[11,28,58,60,79,80], while others have suggested a role for TLR2[7,12,19,37,63]. Initial evidence for TLR4-mediated recognition of *H. pylori* LPS was provided by Kawahara *et al*[79] (2001) who demonstrated that LPS from clinical isolates of *H. pylori* induced increased superoxide anion (O_2^-) production in guinea pig gastric pit cells that ex-

Table 2 Toll-like receptors involved in the response to distinct *Helicobacter pylori* components

H. pylori component	TLR	Ref.
LPS	TLR4	Kawahara *et al*[79]
		Su *et al*[80]
		Ishihara *et al*[58]
		Mandell *et al*[11]
		Chochi *et al*[60]
		Cullen *et al*[28]
	TLR2	Smith *et al*[19]
		Lepper *et al*[63]
		Yokota *et al*[12]
		Triantafilou *et al*[37]
		Smith *et al*[7]
Flagellin	TLR5	Smith *et al*[19]
	TLR5 evasion	Lee *et al*[84]
		Gewirtz *et al*[83]
HSP60	TLR2	Takenaka *et al*[59]
		Zhao *et al*[85]
	TLR2-independent	Gobert *et al*[66]
HP0175	TLR4	Basak *et al*[87]
		Pathak *et al*[65]
		Basu *et al*[86]
NAP	TLR2	Amedei *et al*[6]
H. pylori DNA	TLR9	Rad *et al*[18]
H. pylori RNA	TLR7/TLR8	Rad *et al*[18]

TLR: Toll-like receptor; *H. pylori*: *Helicobacter pylori*.

press endogenous TLR4, but not TLR2. Subsequently, TLR4 antibodies were shown to inhibit LPS-mediated IL-8 secretion from phorbol myristate acetate (PMA)-stimulated THP1 macrophages and *H. pylori* demonstrated increased adherence to Chinese Hamster Ovary (CHO) cells transfected with TLR4 compared with that of CHO-TLR2 or untransfected CHOs[80]. Using reporter gene assays, Ishihara *et al*[58] (2004) described *H. pylori* LPS-mediated NF-κB activation and transcription from the IL-8 promoter in AGS gastric epithelial cells over-expressing TLR4 and MD2[58]. In addition, Mandell *et al*[11] (2004) demonstrated that although TLR2 plays a key role in the response to intact *H. pylori*, TLR4-deficient murine BMDMs were unresponsive to LPS isolated from clinical strains of *H. pylori* with regard to cytokine (IL-6) production. More recently, Chochi *et al*[60] (2008) demonstrated that a clinical isolate of *H. pylori* LPS augmented proliferation using a panel of gastric cancer cell lines (MKN28, MKN45, NUGC3 and KATOIII) in a TLR4-dependent manner. Lastly, while investigating the role of lipid A modifications in *H. pylori* pathogenesis, Cullen *et al*[28] (2011) reported that modification of *H. pylori* LPS in terms of lipid A dephosphorylation leads to decreased LPS-mediated NF-κB activation in HEK-TLR4 cells, providing a mechanism whereby *H. pylori* evades innate immune recognition.

In support of TLR2 as the *H. pylori* LPS receptor, Smith *et al*[19] (2003) demonstrated that LPS isolated from *H. pylori* NCTC 26695 induced NF-κB-dependent reporter gene activity in HEK293 cells transfected with TLR2, but not with TLR4. In addition, LPS from *H. pylori* strain LC11 and two clinical isolates activated NF-κB in HEK-TLR2 cells but not HEK-TLR4 cells. Also using HEK cell lines transfected with TLRs, studies from the Triantafilou laboratory indicated that *H. pylori* LPS induced TNFα production in TLR2-expressing cells, but not TLR4-expressing cells[37,63]. TLR2 was responsible for *H. pylori* LPS-mediated NF-κB-driven reporter gene activity in CHO fibroblasts and HEK cells over-expressing TLR2[37,63]. Inhibition of endogenous TLR2 expression in vascular endothelial cells by RNA interference resulted in a reduction of TNFα production[37]. Using fluorescence resonance energy transfer analysis, they also demonstrated that TLR2 is recruited to lipid rafts and associates with TLR1 in cells following LPS stimulation in vascular endothelial cells[37]. Further, Yokota *et al*[12] demonstrated that *H. pylori* LPS-mediated induction of IL-8 secretion from T24 uroepithelial cells was suppressed by expression of a dominant negative TLR2 mutant, but not with a TLR4 mutant. NF-κB-dependent luciferase reporter assays indicated that over-expression of TLR2 and TLR1 or TLR2 and TLR6 conferred LPS responsiveness in HEK293 cells. The combination of TLR2 and TLR1 expression resulted in higher responsiveness to *H. pylori* LPS than TLR2 and TLR6 expression[12].

Studies by Smith *et al*[7] (2011) have also supported a role for TLR2 in the innate immune recognition of *H. pylori* LPS. LPS prepared from 3 reference strains (NCTC 11637, NCTC 26695 and CCUG 17874) and 4 clinical isolates of *H. pylori* induced IL-8 mRNA expression in HEK293 cells over-expressing TLR2 but not TLR4. IL-8 induction in HEK-TLR2 cells was found to be dose-dependent with a significant level of induction observed at the lowest LPS concentration tested (250 ng/mL). The effect was shown to be LPS specific, as pre-incubation of the *H. pylori* LPS preparations with the antibiotic polymyxin B, a well-known inhibitor of the activating properties of LPS, resulted in a dose-dependent decrease in IL-8 induction in HEK-TLR2 cells[7]. It was also found that *H. pylori* LPS did not induce IL-8 expression in AGS cells, which do not express TLR2 endogenously[20,62], whereas IL-8 was induced in MKN45 cells and T84 colorectal carcinoma cells which have been shown to express endogenous TLR2[19,57,81]. In order to delineate LPS-mediated signalling downstream of TLR engagement, co-transfection using dominant negative constructs and small-interfering RNA demonstrated that *H. pylori* LPS functioned as a classic TLR2 ligand by signalling through pathways involving MyD88, MAL, IRAK1, IRAK4, TRAF6, IKKβ and IκBα to activate NF-κB and transcription form the IL-8 promoter[7]. Through a combination of microarrays, quantitative PCR and ELISAs, it was demonstrated that *H. pylori* LPS induced expression of ICAM1 and the chemokines CXCL1, CXCL2, CXCL3 and CCL20 in TLR2-expressing HEK cells and MKN45 gastric epithelial cells but not HEK293, HEK-TLR4 or AGS cells. Increased expression of these genes was confirmed in gastric tissue biopsy samples from *H. pylori*-infected patients when

compared to uninfected controls[7].

The reasons for the conflicting results between the studies are unclear. Possible explanations include differences in experimental systems involving alternative read-outs for TLR activation and various cell lines from different species. In addition, contamination of the LPS preparation with other components, such as protein, nucleic acids or other bacterial LPS molecules could account for conflicting findings. However, results from Smith *et al*[7] (2011) demonstrating that polymyxin B inhibited TLR2-mediated IL-8 induction in HEK-TLR2 cells would imply that the TLR2-mediated response observed was LPS-specific and not due to the presence of other contaminating TLR ligands, at least in this cell context. Contrasting findings may also have arisen due to heterogeneity of the structures of *H. pylori* LPS molecules resulting from strain differences and/or culturing conditions. Tran *et al*[75] (2005) reported that the lipid A portion of *H. pylori* LPS undergoes several structural modifications through the action of specific modifying enzymes. There is also considerable LPS structural variability due to diversity in the chemical composition of the polysaccharide O-antigen[27,28]. The study by Yokota *et al*[12] (2007) reported similar TLR2-dependent activities using LPS isolated from 6 different clinical isolates of *H. pylori* that demonstrated various characteristics, such as smooth/rough phenotypes and antigenicity of the polysaccharide portion[12]. Additionally, it has been shown that LPS isolated from other gram-negative bacteria that produce a mixture of lipid A species with modified forms of lipid A, such as *Porphyromonas gingivalis* and *Leptospira interrogans*, elicit immune responses through TLR2[33-37].

Flagellin

TLR5 has been identified as the receptor for bacterial flagellin[38], the protein subunit of the polymeric flagellar filament of different gram-positive and gram-negative bacteria. *H. pylori* flagella (5-7 per cell) confer motility and are composed of polymers of two protein subunits, the major flagellin FlaA and the minor flagellin FlaB[82,83], both of which are essential for the bacteria to survive in the stomach musica[84]. TLR5 is expressed on primary gastric epithelial cells and gastric epithelial cell lines, including AGS, HM02, MKN28 and MKN45[19,62,84]. Initial investigations into the innate immune recognition of *H. pylori* flagellin indicated that TLR5 expression in HEK293 cells conferred responsiveness to partially purified flagellin from *H. pylori* in terms of NF-κB-dependent reporter gene activity[19] (Table 2). In addition, transfection of MKN45 cells with a dominant negative TLR5 construct inhibited NF-κB activity in response to *H. pylori* flagellin[19]. However, other studies have since demonstrated that *H. pylori* flagellin is a significantly less potent stimulator of TLR5 signalling than flagellin from other gram-negative bacteria, such as *Salmonella typhimurium*[83,84]. Lee *et al*[84] (2003) demonstrated that although IL-8 release induced by *H. pylori* with mutations in one or both flagellins was delayed compared to wild type *H. pylori*, purified native or recombinant flagellins did not significantly stimulate IL-8 secretion from gastric epithelial cells despite the presence of TLR5, suggesting that the delayed effect with the mutant strains may have been a result of decreased bacterial motility or adherence. Gewirtz *et al*[83] (2004) found no impairment in the IL-8 inducing ability of *H. pylori* in AGS cells as a result of FlaA mutations compared to the wild type strain. In keeping with the findings of Lee *et al*[84] (2003), purified *H. pylori* flagellin failed to induce significant innate immune responses in gastric epithelial cells as assessed by p38 MAPK induction and IL-8 secretion. The low innate immune response to *H. pylori* flagellin in the stomach *in vivo* may provide another mechanism that contributes to the ability of *H. pylori* to evade host responses and to promote long term bacterial persistence.

Heat shock protein 60

The 60 kDa heat-shock protein (HSP60) of *H. pylori* plays a role in the adherence and attachment of *H. pylori* to the gastric epithelium and is a potent immune antigen that stimulates IL-8 induction in gastric epithelial cells[59]. HSP60-induced immune responses are associated with gastric inflammation and the pathogenesis of MALT[59]. Takenaka *et al*[59] (2004) have suggested that *H. pylori* HSP60 is a TLR2 ligand as HSP60-mediated NF-κB activation and IL-8 production in KATO Ⅲ human gastric epithelial cells was inhibited using a TLR2 blocking antibody (Table 2). *H. pylori* HSP60 has also been shown to induce IL-8 production in human monocytes. In support of TLR2 in the recognition of *H. pylori* HSP60, Zhao *et al*[85] (2007) reported that treatment of NOMO1 human monocytes with an anti-TLR2 blocking antibody or small interfering RNA for TLR2 inhibited NF-κB, ERK and p38 MAPK activation as well as IL-8 secretion in response to recombinant *H. pylori* HSP60 stimulation. In contrast to the findings in human cells, peritoneal macrophages from mice deficient in TLR2, TLR4, MyD88 or both TLR2 and TLR4 produced the same amount of IL-6 in response to *H. pylori* HSP60 as wild type macrophages, indicating TLR-independent IL-6 induction in this cell context[66].

H. pylori peptidyl prolyl cis-,trans-isomerase HP0175

H. pylori secretes the peptidyl prolyl *cis*-, *trans*-isomerase HP0175, which can induce apoptosis in gastric epithelial cells and is one of the highly and consistently reactive *H. pylori* antigens recognized in the sera of *H. pylori*-infected patients[65,86]. Studies from the Kundu laboratory have described a role for TLR4 in the recognition of HP0175 (Table 2). Initially, Basak *et al*[87] (2005) demonstrated interaction between TLR4 and HP0175 in AGS cells using pull-down immunoassays. Inhibition of TLR4 using a neutralizing antibody or a dominant negative construct inhibited HP0175-induced apoptosis in AGS cells[87]. Pathak *et al*[65] (2006) subsequently reported that HP0175 induced the release of IL-6 from PMA-differentiated

THP1 macrophages, whereas isogenic mutants of *H. pylori* 26695, in which the *Hp0175* gene was disrupted, elicited decreased IL-6 production. A role for TLR4 in this process was suggested because pre-treatment of cells with a TLR4 (but not TLR2) neutralising antibody or transfection with a dominant-negative TLR4 construct blocked HP0175-mediated IL-6 release. In addition, TLR4 expression (but not TLR2) in HEK293 cells conferred responsiveness to HP0175. Using ELISA-based binding assays, Pathak *et al*[65] also showed that HP0175 interacts with the extracellular domain of TLR4 in the absence of any accessory molecules. Finally, Basu *et al*[86] (2008) showed that HP0175 transactivates the epidermal growth factor receptor (EGFR) and stimulates EGFR-dependent vascular endothelial growth factor (VEGF) production in AGS cells in a TLR4-dependent manner.

NapA

The *H. pylori* neutrophil-activating protein (NAP) is a 150 kDa oligomeric virulence factor that is chemotactic for neutrophils, stimulates high production of oxygen radicals in neutrophils and their adhesion to endothelial cells. Amedei *et al*[6] (2006) have reported that the *H. pylori* NAP is a TLR2 agonist, because over-expression of TLR2 in HEK293 cells resulted in NAP-mediated NF-κB-dependent reporter gene activity (Table 2). NAP stimulation had no effect on untransfected HEK293 cells, or HEK293 cells over-expressing TLR3, TLR4, TLR5, TLR7, TLR8 or TLR9. In human monocytes and neutrophils, NAP stimulation induced the expression of IL-12[6], which is an important cytokine for the differentiation of naïve Th cells into the Th1 phenotype. NAP also induced monocytes to produce IL-23 and differentiate towards mature DCs[6]. Stimulation of antigen-induced T-cells with NAP resulted in increased numbers of IFN-γ-producing T cells and decreased numbers of IL-4-secreting cells, thus promoting a Th1 phenotype. In addition, using T cell clones generated from *in vivo*-activated T cells derived from the gastric mucosa of *H. pylori*-infected patients, NAP was shown to elicit Th1-polarizing capacity[6], implying that the TLR2: *H. pylori* NAP interactions promote the activation of innate immunity to drive IL-12 and IL-23 production and the subsequent promotion of Th1 immune responses.

Nucleic acids

TLR9 recognises unmethylated CpG DNA in bacteria[40] and also detects herpes virus DNA[41]. TLR7 and TLR8 have been shown to sense single-stranded viral RNA[42-44]. Rad *et al*[18] (2009) have demonstrated TLR9-mediated recognition of *H. pylori* DNA in DCs and the subsequent induction of pro-inflammatory cytokine secretion. They showed that IL-6 and IL-12 production was completely abrogated in TLR2/TLR4/TLR9-deficient BMDCs compared to TLR2/TLR4-deficient cells in response to purified *H. pylori* DNA following pre-treatment with ribonuclease. Expression of TLR9 is increased in mouse gastric tissue following *H. pylori* infection and is mainly localised to macrophages, DCs and $CD3^+$ cells in the gastric mucosa[88]. Although purified *H. pylori* DNA was reported to induce a TLR9-mediated increase in IL-6 and IL-12 expression in BMDCs[18], in a mouse model of *H. pylori* infection TLR9 signalling was shown to have an anti-inflammatory effect on the early phase of *H. pylori*-induced gastritis as genetic disruption of TLR9 resulted in an increase in *H. pylori*-induced gastric mucosal inflammation characterized by neutrophil infiltration and increased expression of TNFα and IFNγ[88]. In relation to TLR7 and TLR8-mediated recognition of *H. pylori*, Rad *et al*[18] (2009) showed that purified *H. pylori* RNA (pre-treated with deoxyribonuclease) induced pro-inflammatory cytokines in BMDCs in a MyD88-dependent manner involving the endosomal TLR8, possibly in collaboration with TLR7.

TARGETING TLR SIGNALLING THERAPEUTICALLY

As TLRs are intimately involved in the regulation of inflammation during innate immunity and represent key activators of adaptive immunity, they represent an attractive therapeutic target for treatment of inflammatory diseases. Indeed, oligonucleotide inhibitors of TLR7 and/or TLR9 have been shown to have therapeutic potential in animal models of systemic lupus erythematosus[89]. Additionally, an inhibitory TLR2 antibody was demonstrated to limit ischemia-reperfusion injury in the hearts of pigs[90] and kidneys of mice[91]. In the clinic, therapies involving the synthetic small molecule inhibitor of TLR4, Eritoran (also known as E5564), were used in trials for patients with sepsis, but only had marginal effects possibly as treatment was administered too late following disease onset[92,93]. TLR activation is also important for adjuvancy in vaccines and several TLR ligands have been shown to be efficacious as vaccine adjuvants[94,95]. For example, the vaccine adjuvant monophosphoryl lipid A, which is a less toxic version of LPS, promotes antibody responses via TLR4 activation[96]. Efficient preventative or therapeutic vaccination for *H. pylori* has not been achieved in humans to date[97]. Early signs of promise in animal models of *H. pylori* infection have been unsuccessful in humans. In a recent study to investigate the vaccine potential of *H. pylori* LPS, Altman *et al*[98] (2012) demonstrated enhanced antibody responses to a chemically modified LPS in mice and rabbits and partial protection against *H. pylori* challenge, warranting further investigation in this area. In terms of adjuvancy, immunization against *Helicobacter* using CpGDNA and cholera toxin demonstrated synergism leading to sterile immunity in mice[99]. More recently, Mori *et al*[100] (2012) constructed a chimeric flagellin by replacing the N- and T-terminal segments of *H. pylori* flagellin with a TLR5-stimulating adjuvant component of *E. coli* flagellin in order to enhance innate and adaptive immunity. The resulting chimeric flagellin activated TLR5 signalling and elicited a strong antibody response in mice. Together

with alum, vaccination with the chimeric flagellin protected mice from *H. pylori* infection[100]. In other disease settings, local administration of the TLR2 ligand *H. pylori* NAP was demonstrated to decrease tumour growth by activating a cytotoxic Th1 response in a mouse model of bladder cancer[101]. Taken together, these studies indicate that defining *H. pylori*-derived molecules responsible for TLR activation and elucidating innate immune signals triggered by *H. pylori*, may provide insight into the design and development of novel human vaccine adjuvants and therapeutics.

H. PYLORI RECOGNITION BY OTHER PRRS

Microbial pathogens activate multiple PRRs and different PRRs may recognize the same PAMP within an organism[17]. Insight into the co-operation between TLRs and other PRRs during infection is necessary for a complete understanding of the innate immune response during infection. These PRRs include nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) and C-type lectin receptors (CLRs). *H. pylori* peptidoglycan, delivered either by the type IV secretion system or through outer membrane vesicles secreted from the bacterium, is recognised by NOD1 in epithelial cells[102-107]. Moreover, studies by Kim *et al*[70] (2013) have described the cooperative interaction of TLR2 and NOD2 in the regulation of IL-1β in *H. pylori*-infected DCs. In addition to a role for TLR8 in the response to *H. pylori* RNA, findings by Rad *et al* (2009) indicated that *H. pylori* induces expression of type I interferon and interferon-stimulated genes in a MyD88- and TRIF-independent manner and demonstrated that the MyD88-independent type I IFN induction by *H. pylori* RNA was mediated by RIG-I[18]. In relation to *H. pylori*-mediated CLR signalling, Gringhuis *et al*[108] (2009) have reported that the fucose residues of *H. pylori* DC-SIGN ligands actively disrupt signalling down-stream of DC-SIGN to suppress pro-inflammatory cytokine induction.

CONCLUSION

Although, *H. pylori* induces a strong immune response, elimination of infection is not achieved. The pathogenesis of *H. pylori*-associated disease is linked to the severity of the host inflammatory response**.** Emerging evidence suggests that failure to eliminate *H. pylori* may be due to the ability of the bacterium to control T-cell responses. As TLRs are intimately involved in the regulation of inflammation during the innate immune response to *H. pylori* and represent key activators of adaptive immunity, a significant effort has been made to elucidate their role in the recognition of *H. pylori* and its components in multiple cell types. Much of the literature has focussed on the involvement of individual TLRs in the induction of pro-inflammatory cytokines in various *in vitro* cell culture models. There is substantial evidence to support the role of TLR2 in activating NF-κB or inducing cytokine expression in response to *H. pylori* infection in epithelial cells[7,11,19,20], monocytes/macrophages[11,67], dendritic cells[18,70,73] and B cells[21]. Numerous *H. pylori* ligands have been suggested to date that may contribute to these TLR2-dependent responses, including LPS[7,12,19,37,63], HSP60[59,85] and NAP[6]. TLR4 has also been implicated in the response to *H. pylori*[18,57,67], which may be mediated by LPS[11,28,58,60,79,80] and/or HP0175[65,86,87]. TLR9 has been identified as the receptor for *H. pylori* DNA[18]. Although *H. pylori* flagellin has been suggested as a TLR5 ligand[19], its activity as a TLR5 activator is low[83,84], providing a possible mechanism that contributes *H. pylori* persistence.

Recent studies using mouse models of infection have provided insight into the role of TLR signalling in regulating *H. pylori*-mediated T cell responses, gastric immunopathology and colonization *in vivo*. Interestingly, although a demonstrated role for TLR2 in the induction of pro-inflammatory cytokines has been described in distinct cell populations, the net effect of TLR2 signalling has been reported to mediate tolerance and promote bacterial persistence in mouse models of infection by skewing T cell responses[21,73]. Further *in vivo* studies elucidating innate immune signals triggered by *H. pylori*-mediated activation of TLRs, especially in cooperation with other PRRs, are necessary for a complete understanding of how the balance between pro-inflammatory and anti-inflammatory signals fine-tunes the immune response to *H. pylori* infection, and may provide insight into how the immune response may be manipulated therapeutically to successfully eradicate the bacterium.

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TOPIC HIGHLIGHT

WJGP 5th Anniversary Special Issues (3): Pancreatitis

Molecular mechanisms of alcohol associated pancreatitis

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Abstract

Alcohol abuse is commonly associated with the development of both acute and chronic pancreatitis. Despite this close association, the fact that only a small percentage of human beings who abuse alcohol develop pancreatitis indicates that alcohol abuse alone is not sufficient to initiate clinical pancreatitis. This contention is further supported by the fact that administration of ethanol to experimental animals does not cause pancreatitis. Because of these findings, it is widely believed that ethanol sensitizes the pancreas to injury and additional factors trigger the development of overt pancreatitis. How ethanol sensitizes the pancreas to pancreatitis is not entirely known. Numerous studies have demonstrated that ethanol and its metabolites have a number of deleterious effects on acinar cells. Important acinar cells properties that are affected by ethanol include: calcium signaling, secretion of zymogens, autophagy, cellular regeneration, the unfolded protein response, and mitochondrial membrane integrity. In addition to the actions of ethanol on acinar cells, it is apparent that ethanol also affects pancreatic stellate cells. Pancreatic stellate cells have a critical role in normal tissue repair and the pathologic fibrotic response. Given that ethanol and its metabolites affect so many pancreatic functions, and that all of these effects occur simultaneously, it is likely that none of these effects is "THE" effect. Instead, it is most likely that the cumulative effect of ethanol on the pancreas predisposes the organ to pancreatitis. The focus of this article is to highlight some of the important mechanisms by which ethanol alters pancreatic functions and may predispose the pancreas to disease.

Key words: Pancreatitis; Alcoholic pancreatitis; Alcoholic acute pancreatitis; Alcoholic chronic pancreatitis

Core tip: Alcohol abuse is commonly associated with the development of acute and chronic pancreatitis. Despite this close association, the fact that only a small percentage of human beings who abuse alcohol develop pancreatitis indicates that alcohol abuse alone is not sufficient to initiate clinical pancreatitis. It is widely believed that ethanol sensitizes the pancreas to injury and additional factors trigger the development of overt pancreatitis. How ethanol sensitizes the pancreas to pancreatitis in not entirely known. We will review the mechanisms by which ethanol is thought to sensitize human beings to pancreatic injury.

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INTRODUCTION

The pancreas is a complex organ, containing both exo-

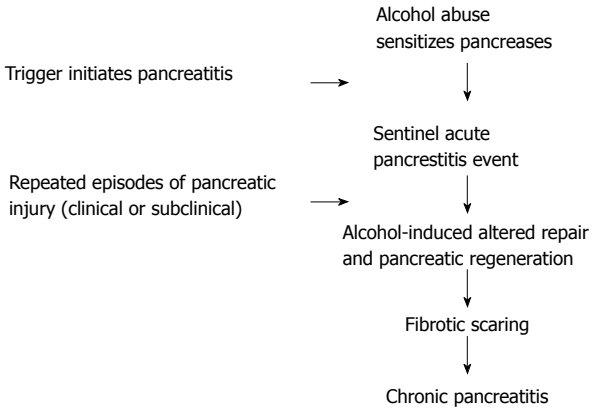

Figure 1 Proposed model for the development of alcoholic chronic pancreatitis. This proposed model incorporated alcohol abuse into the seminal acute pancreatitis event (SAPE) model proposed by Whitcomb. Alcohol metabolism results in biochemical and molecular changes in acinar cells that sensitizes the pancreas to injury. A secondary trigger initiates an initial episode of acute pancreatitis. This is the SAPE. Repeated clinical or subclinical episodes of pancreatitis coupled with ethanol-induced aberrant repair and regeneration of the damaged pancreas leads to fibrotic scarring which eventually results in chronic pancreatitis.

crine and endocrine components. The endocrine component of the pancreas comprises only about 1%-2% of the organ, and is responsible for the production of insulin and glucagon, both of which regulate glucose homeostasis. The exocrine component comprises the vast majority of the pancreas; it is composed of acinar, stellate, and ductal cells. The acinar cells produce digestive enzymes, which facilitate the digestion of carbohydrates, proteins, and lipids. The ductal cells form a network that serves as a conduit for delivery of these enzymes into the duodenum. The pancreatic stellate cells synthesize and degrade extracellular matrix proteins.

Pancreatitis, or inflammation of the pancreas, is a necroinflammatory disease of the pancreas that can manifest as either an acute or chronic disease. Acute pancreatitis is characterized by various degrees of acinar cell damage with concomitant local and systemic inflammation, mediated by inflammatory cytokines and chemokines[1]. Acute pancreatitis is usually a self-limiting condition. Unfortunately, in 10% to 20% of clinical cases, acute pancreatitis progresses to severe acute pancreatitis, a disease with high morbidity and mortality. In the United States alone there are approximately 210000 new clinical cases of acute pancreatitis a year[2]. In 2009, acute pancreatitis was the most common gastrointestinal disease requiring hospitalization. Additionally, it was estimated that acute pancreatitis accounted for more than 2.5 billion dollars in direct and indirect costs[3]. Obviously, pancreatitis is a serious public health concern.

Chronic pancreatitis is a progressive disease characterized by severe pain, persistent pancreatic inflammation, and the development of fibrotic scarring, as well as the loss of endocrine and exocrine function. It has been demonstrated in a long-term prospective study that alcoholic chronic pancreatitis normally progresses from acute pancreatitis. Additionally, this study demonstrated that the progression of acute pancreatitis to chronic pancreatitis is associated with the frequency and severity of the acute attacks[4]. These findings are supported by the observation that individuals who suffer frequent attacks of acute pancreatitis progress to chronic pancreatitis more rapidly[5]. These findings led Whitcomb to propose that a sentinel acute pancreatitis event (SAPE) is required for the development of chronic pancreatitis[6] (Figure 1). Therefore, it appears that although acute and chronic pancreatitis have different clinical manifestations, the mechanisms by which the disease process is initiated is likely similar[7]. Unfortunately, there currently is no treatment, other than palliative care, for either of these diseases.

One of the most common factors associated with both acute and chronic pancreatitis is alcohol abuse[8]. In fact, the association between alcohol abuse and pancreatic disease has been recognized for well over 100 years[9]. It has been known for sometime that the risk of developing pancreatitis increases with increasing alcohol consumption. Recent studies have shown that a threshold of approximately 5 drinks/d (60 g of ethanol) is required for significantly increased risk of developing pancreatitis[10-12]. Although numerous studies have demonstrated direct toxic effects of ethanol and its metabolites on the pancreas, the majority of heavy drinkers (even those consuming more than 5 drinks a day) do not develop pancreatitis[8,12,13]. This fact clearly indicates that alcohol abuse itself is not sufficient to cause pancreatitis, and an additional insult or additional factors are required for the development of clinical pancreatitis. Among the factors suggested to be involved in alcoholic pancreatitis are: smoking, high fat diet, obesity, genetics, and infectious agents[12-16].

Despite the long-standing recognition of the association between alcohol and pancreatitis, the biochemical and molecular processes by which ethanol influences the initiation and progression of these diseases is not well understood. It is thought that the toxic effects of ethanol and/or the by-products of ethanol metabolism sensitize the pancreas; thereby, lowering the threshold to damage from other factors. Ethanol has been shown to affect a number of pathways and functions important in acinar cells. Alteration of these pathways may individually or cumulatively sensitize the pancreas, and lower the threshold of the pancreas to the development of overt pancreatitis. Ethanol has been shown to affect a number of pathways and functions important in acinar cells (Table 1).

Both the rapid course of acute pancreatitis and the relative inaccessibility of pancreatic tissue for examination, prior to the development of fibrotic damage in chronic pancreatitis, have hampered detailed investigations using tissue from human beings. This has contributed to our limited understanding of the mechanisms that lead to the initiation and the progression of alcoholic pancreatitis. Because of this, much of our understand-

Table 1 Mechanisms by which ethanol is thought to sensitize the pancreas to pancreatitis

Alteration of cell death pathways
Altered vesicular trafficking
Impaired autophagy
Impaired tissue repair
ER stress
Mitochondrial dysfunction

ing of pancreatitis in general, and alcoholic pancreatitis in particular, has come from the use of preclinical animal models. Preclinical models used to investigate alcoholic pancreatitis normally utilize mice or rats administered ethanol. Ethanol administration to experimental animals is commonly accomplished through the Tsukamoto-French intragastric method[17], the Lieber-DeCarli pair feeding method[18], or the Cook-Meadows model of providing ethanol in the drinking water[19,20]. Pancreatic cells are either isolated from the animals administered ethanol or pancreatitis is induced. Among the more common methods of inducing pancreatitis in these animals are: bile duct ligation, treatment with supraphysiological concentrations of the cholecystokinin (CCK) analogue caerulein, or treatment with trinitrobenzene sulfonic acid (TNBS)[21]. More recently, methods designed to be more clinically relevant have been reported. These methods include chronic ethanol administration followed by treatment with gram-negative bacterial lipopolysaccharide (LPS)[22,23], or infection with Coxsackievirus CVB3[16,24,25].

Unfortunately, no animal model of chronic pancreatitis recapitulates all of the manifestations of chronic pancreatitis in human beings. It has been demonstrated that alcohol administration to rats and mice results in acinar cell loss and enhanced fibrosis in animals subjected to caerulein-induced pancreatic injury[26,27]. Therefore, these models may be useful in elucidating the mechanisms by which ethanol alters normal pancreatic repair, and predisposes the pancreas to fibrosis.

It is the focus of this article to review and highlight some of the molecular events that may adversely affect the pancreas, and sensitize the pancreas to the initiation or progression of alcoholic pancreatitis.

ETHANOL METABOLISM

Many of the deleterious effects of ethanol are attributed to the by-products produced during its metabolism. Like the hepatocytes of the liver, the pancreatic acinar cells have the ability to metabolize ethanol by both oxidative and nonoxidative pathways. The oxidative metabolism of ethanol is catalyzed by two enzymes: the cytosolic enzyme, alcohol dehydrogenase, and the microsomal enzyme, cytochrome P450 2E1. Ethanol metabolism by both of these enzymes generates acetaldehyde and reactive oxygen species. Although the pancreas expresses both alcohol dehydrogenase and cytochrome P450 2E1, the capacity for ethanol oxidation by the pancreas is significantly less than that of the liver[28,29]. Therefore, the actions of the oxidative metabolites of ethanol oxidation may result from both pancreatic metabolism and systemic metabolism of ethanol.

Nonoxidative metabolism of ethanol is carried out by a number of enzymes, the most important being the fatty acid ethyl ester synthases. Metabolism of ethanol by these enzymes generates fatty acid ethyl esters (FAEEs). The pancreas possesses high fatty acid ester synthase activity. Thus, the capacity for nonoxidative metabolism of ethanol in the pancreas is high[30]. In fact, a study of individuals who were intoxicated at the time of death revealed that the concentration of FAEEs in the pancreas was higher than any other organ analyzed[30]. Thus, because the oxidative metabolism of ethanol in the pancreas is relatively low, the nonoxidative metabolism of ethanol may be more important and the production of FAEEs, and their toxic effects, may be accentuated. Because the by-products of ethanol metabolism have been demonstrated to cause toxicity in other organs, a great deal of work has been performed investigating the actions of the various ethanol metabolites on the pancreas.

CELL DEATH

Cell death during an episode of acute pancreatitis can occur by one of two mechanisms: apoptosis or necrosis. The distinction between the two types of cell death not only has biological implications in the development of acute pancreatitis, but also affects the clinical presentation by influencing the severity of the illness[8]. Clinically, according to the 2012 Atlanta Classification of Acute Pancreatitis, the presence of necrosis and the number of organs affected by the subsequent inflammatory response determines the severity of acute pancreatitis (mild, moderate, severe) and dictates the short-term and long-term management of these patients[31].

While necrosis and apoptosis both lead to cell death, their respective mechanisms of achieving this end are quite different. Apoptosis, or programmed cell death, is a process by which cellular constituents are cleaved by cysteine-dependent, aspartate-directed enzymes, known as caspases. Apoptosis is mediated by caspases 3 and caspases 8. Caspase 8 is the initiator of the caspase cascade and cleaves caspase 3, which mediates many of the cellular changes that lead to apoptotic death. In pancreatitis, these caspases are activated by the release of cytochrome c from mitochondria[32]. The release of cytochrome c is caused by the depolarization of mitochondria. It appears this depolarization is a result of the opening of the mitochondrial permeability transition pore, which is caused by sustained increased calcium levels in the cytosol[33]. Ultimately, there is an organized dismantling of the cell. This leads to cell shrinkage and nuclear chromatin condensation, while preserving the integrity of the plasma membrane. Because the plasma membrane remains intact, there is very little leakage of intracellular material into the extracellular space, and therefore; there is little

activation of inflammatory cytokines.

In contrast to the organized dismantling of the cell in apoptosis, necrosis involves intracellular swelling of organelles and rupture of the plasma membrane. This results in the release of the contents of the cell into the extracellular space, which causes an inflammatory response. It has been shown in a number of preclinical animal models of pancreatitis that the severity of pancreatitis is increased with increasing necrotic cell death[8]. Additionally, perhaps the most important prognostic indicator of the severity of pancreatitis in human beings is the amount of necrosis[31].

In preclinical animal models of pancreatitis, ethanol has been shown to cause a shift in cell death from apoptosis to necrosis. This shift has been shown to occur through several mechanisms. It has been shown that the nonoxidative metabolites of ethanol, FAEEs, activate inositol trisphosphate receptors on the endoplasmic reticulum. Activation of these receptors causes release of calcium into the cytosol. As stated above, the sustained increases in cytosolic calcium results in mitochondria depolarization and loss of ATP production. Without ATP, the cells are unable to complete the apoptotic process and necrosis occurs[8].

Ethanol has also been shown to inhibit the JAK2/STAT1 pathway. Attenuated activity of this pathway leads to decreased activity of both caspase 8 and caspase 3[32]. With lower activity of these caspases, cell death by necrosis is increased while apoptotic cell death is reduced.

Ethanol also increases the pancreatic expression of cathepsin B[32]. Cathepsin B is a cysteine protease that is thought to play a major role in the intrapancreatic conversion of trypsinogen to trypsin. It has been shown that in pancreata of ethanol-fed rats, increased expression of cathepsin B result from increased levels of the transcriptional activators Ets-1 and Sp1[32]. Increases in Sp1 and Ets-1 enhance expression of cathepsin B, which leads to activation of trypsin and a shift from apoptosis to necrosis in pancreatic acinar cells[32]. These findings demonstrate that ethanol can affect the mechanism of cell death in acinar cells, and thereby influence the severity of the disease.

EFFECTS OF ETHANOL ON ZYMOGEN SECRETION

One of the primary roles of the exocrine pancreas is the synthesis and secretion of digestive enzymes. The pancreas is protected from the actions of these potentially dangerous enzymes because they are synthesized as inactive zymogens and packaged into exocytotic vacuoles, known as zymogen granules. Although ethanol has many effects on acinar cells that contribute to the development of pancreatitis, the inappropriate activation of zymogens is likely a critical component of this pathologic process.

Activation of trypsinogen is generally considered a pivotal event in the initiation of pancreatitis[34]. It has been reported by Gorlelich that treatment of isolated acinar cells with intoxicating concentrations of ethanol (25 mmol/L) sensitizes acinar cells to damage by causing the activation of zymogens[35]. The activation of these zymogens required an increase in cytosolic calcium and appeared to involve a low pH compartment (acid granular compartment).

Local cytosolic spikes of calcium in the apical region of acinar cells control the exocytotic secretion of zymogens. These spikes are generated by release of small quantities of calcium from internal stores[36]. In contrast, prolonged, global elevation of calcium results in the formation of empty looking zymogen granules, this is thought to be the site where trypsin is activated. In acinar cells treated with the FAEE palmitoleic acid ethyl ester, calcium was released from both the endoplasmic reticulum (the major calcium storage compartment of the cell) and the acid granular compartment, located near the apical surface. Additionally, it was demonstrated that the calcium release was primarily mediated by type 2 and 3 inositol 1,4,5 trisphosphate receptors[37].

Normally, zymogens are released from acinar cells by fusion of zymogen granules with the apical membrane. This fusion results in their release into the ducts, where they are transported to the duodenum and activated. The components absolutely required for membrane fusion consist of: SNAREs (soluble NSF [N-ethylmaleimide-sensitive fusion proteins] attachment proteins receptors) located on the target membrane, t-SNARES, and v-SNAREs, also known as vesicle-associated membrane proteins (VAMPs), located on the membrane of the vesicle. The t-SNARES syntaxin and synaptosome-associated proteins (SNAPs), form a SNARE complex that binds to its cognate v-SNARE; thus, juxtaposing the two membranes and facilitating the fusion of the membranes.

Interestingly, it has been demonstrated both *in vivo* and *in vitro,* that supramaximal treatment with cholecystokinin (CCK) causes basolateral exocytosis of zymogen granules in acinar cells[38]. Additionally, in both ethanol-fed rats or isolated acinar cells treated with physiologic concentrations of ethanol (20 mmol/L), stimulation with submaximal concentration of CCK or carbachol resulted in the exocytosis being redirected from the apical surface, where zymogens are normally secreted, to the basolateral surface[39]. The authors postulate that the ensuing ectopic activation of the zymogens in the interstitial space results in pancreatitis[39]. More detailed investigations demonstrated that this inappropriate exocytosis was mediated by phosphorylation of mammalian uncoordinated-18c (Munc 18c) by protein kinase C-alpha (PKC-α). Phosphorylation of Munc-18c results in its release from syntaxin-4, which is located on the basolateral surface of acinar cells. Syntaxin-4 is then able to complex with SNAP-23 and VAMP-8, located on the zymogen granules, to form the SNARE complex, which mediates the inappropriate basolateral exocytosis of zymogens[40]. Importantly, basolateral exocytosis has been

observed in tissue samples from a patient suffering from chronic alcoholic pancreatitis[41].

IMPAIRMENT OF AUTOPHAGY

Autophagy is a cellular process in which unnecessary or damaged cellular components or organelles are sequestered in vacuoles and transported to the lysosomes. Upon fusion with the lysosomes, the contents of the autophagic vacuoles, the autophagosomes, are degraded. Not only does this process perform an important role in ridding cells of unneeded components, but during times of low nutrient availability autophagy can provide the cell with needed constituents.

Impaired autophagy has been implicated in the pathogenesis of many diseases, including pancreatitis[15,42-45]. Importantly, it has been shown that ethanol can alter the process of autophagy in a number of organs, including the pancreas[43,46,47].

One of the histological hallmarks of pancreatitis is the accumulation of large vacuoles within acinar cells[48]. In a number of preclinical animal models of pancreatitis, as well as in tissue from a patient with acute pancreatitis, it has been demonstrated that these vacuoles are in fact autophagic vacuoles[44,45]. Further investigation revealed that these vacuoles possessed markers of both autophagosomes and lysosomes, and contained undegraded or partially degraded cellular material[45]. These findings indicate that at least the very late events in the autophagic process, namely the degradation of the components of the autolysosomes, are impaired during pancreatitis[45]. Thus, autophagy is activated during pancreatitis, and it appears that the impairment in the ability to complete this process is responsible for the vacuolization characteristic of this disease.

As mentioned above, trypsin activation is thought to be an early event in the initiation of pancreatitis. How this activation occurs is not well understood. It is generally thought that cathepsin B, is mis-sorted to the zymogens granules, where it co-localizes with trypsinogen. Subsequent cleavage of trypsinogen by cathepsin B results in the production of active trypsin. How trypsinogen and cathepsin B come in contact has always been a mystery. It now appears that the impairment in the completion of the autophagy may have a role in the co-mingling of these two enzymes.

Cathepsin L is an enzyme that degrades trypsinogen and trypsin, and cathepsin B is an enzyme that cleaves trypsinogen forming active trypsin. The two are important lysosomal hydrolases. During pancreatitis, increased levels of these enzymes are found in the zymogen granule fraction. Additionally, in alcoholic pancreatitis, as well as other forms of acute pancreatitis, the processing and activation of cathepsin L and cathepsin B is impaired[45,49]. Furthermore, it appears that the impairment in cathepsin L activity is more severe than the impairment in cathepsin B activity, particularly in the zymogen granule fraction[45]. Importantly, zymogen granules were detected in the autophagosomes/autoloysosomes. The authors propose that it is in these autophagosomes/autoloysosomes that trypsinogen and cathepsin B come in contact[45]. The imbalance between cathepsin B and cathepsin L activity in these vacuoles would favor the activation of trypsin, and the initiation of pancreatitis. Thus, impairment in the completion of the autophagic process and subsequent increase in autolysosomes may contribute not only to the accumulation of vacuoles, but also to the inappropriate intracellular activation of trypsin and the initiation of pancreatitis.

Ethanol has been shown to impair other aspects of autophagy. Using a model of alcoholic pancreatitis in which rats were chronically fed ethanol and then treated with LPS to induce acute pancreatitis, Fortunato *et al*[43] demonstrated that in the pancreata of these animals fusion of autophagosomes with the lysosome was impaired. Additional studies demonstrated that Lamp-2, a lysosomal membrane protein required for the fusion of autophagosomes with lysosomes, was depleted in the pancreata of rats suffering from alcoholic pancreatitis[43,50]. Furthermore, analysis of pancreata from human beings revealed that Lamp-2 was also decreased in the pancreata of patients suffering from chronic alcoholic pancreatitis. These results indicate that the ethanol-mediated reduction in lysosomal proteins, particularly Lamp-2, and subsequent impairment in autophagy may be a contributing factor to alcoholic pancreatitis in human beings. Although not investigated, the authors speculated that disruption in the autophagic pathway may contribute to bioenergenic failure in mitochondria. Lack of mitochondrial ATP would favor necrosis, as opposed to apoptosis. Necrotic cell death would cause inflammation and lead to the initiation of pancreatitis[43].

MITOCHONDRIAL DYSFUNCTION

Pancreatic acinar cells are among the most synthetically active cells in the body[51]. This synthetic activity requires a great deal of energy. Because of this, acinar cells contain an inordinate number of mitochondria. Thus, the actions of toxins, such as ethanol, that affect mitochondria can dramatically affect acinar cells.

Normally, acetylcholine or cholecystokinin bind to G-protein linked receptors that are located on the plasma membrane of acinar cells and stimulate the production of secondary messengers. The secondary messengers bind to inositol trisphosphate or ryanodine receptors located on the endoplasmic reticulum, zymogen granules, and endo-lysosomes. This binding results in the transient release of free calcium. Mitochondria take up this calcium, which results in their activation, the synthesis of ATP, and the secretion of zymogens.

Aberrant calcium signaling has long been considered an important factor in the initiation of pancreatic injury[52]. Pathological calcium signaling in acinar cells results from prolonged global release of calcium from the endoplasmic reticulum, as well as zymogen granules and

endo-lysosomes. In fact, early acinar cell injury (vacuolization, trypsin activation, and basolateral zymogen secretion) does not occur without prolonged, sustained release of calcium[53].

Both nonoxidative and oxidative metabolism of ethanol has been shown to contribute to mitochondrial dysfunction and acinar cell death. FAEEs, the nonoxidative metabolites of ethanol, have been shown to cause pancreatic injury by affecting calcium signaling in acinar cells[54,55]. FAEEs increase the intracellular concentration of calcium to toxic levels. This calcium increase is mediated by activation of the inositol trisphosphate receptors located on the endoplasmic reticulum, and results in global sustained increase in intracellular calcium, which causes mitochondrial membrane permeability. Mitochondrial membrane permeability can lead to cell death by either apoptosis or necrosis[56,57].

Mitochondrial membrane permeability results from opening of the mitochondrial permeability transition pore. The mitochondrial permeability transition pore is thought to have at least three major components, the voltage dependent anion channel (VDAC) located on the outer mitochondrial membrane, adenine nucleotide translocase (ANT) located in the inner mitochondrial membrane and cyclophlin-D located within the mitochondrial matrix[53].

One of the important consequences of the opening of the mitochondrial permeability transition pore and mitochondrial membrane permeability can be loss of the mitochondrial membrane potential ($\Delta\Psi M$). Loss of the $\Delta\Psi M$ results in the decreased ability of TP.

Depleted levels of ATP exacerbate the cells ability to regulate calcium by inhibiting the activity of the important ATP-dependent calcium pumps, the sacroplasmic/endoplasmic reticular calcium ATPase (SERCA) located on the ER, and the plasma membrane calcium ATPase (PMCA) located on the plasma membrane. Thus, mitochondrial membrane permeability can exacerbate the dysregulation of calcium homeostasis and lead to acinar cell necrosis.

The oxidative metabolism of ethanol also has deleterious effects on pancreatic mitochondria. Oxidative metabolism of ethanol by alcohol dehydrogenase requires oxidized nicotinamide adenine dinucleotide (NAD^+) as a cofactor, and results in the production of acetaldehyde and reduced nicotinamide adenine dinucleotide (NADH)[58,59]. Acetaldehyde is then metabolized to acetate, primarily by the mitochondrial enzyme aldehyde dehydrogenase-2. Importantly, this reaction also requires NAD^+ as a cofactor, and also results in the production of NADH[58,59]. Thus, metabolism of acetaldehyde to acetate further depletes the availability of NAD^+.

Using isolated acinar cells treated with ethanol, Shalbueva *et al*[60] demonstrated that ethanol treatment led to a decrease in the NAD^+/NADH ratio. This reduction in NAD^+ resulted in activation of the mitochondrial permeability transition pore, mitochondrial depolarization, ATP depletion, and eventually cellular necrosis[60]. Furthermore, their studies revealed that the ethanol oxidation-mediated polarization of pancreatic mitochondria was attenuated in acinar cells isolated from mice deficient in cyclophilin-D. These results indicate a role for cyclophilin-D in this ethanol metabolism-mediated mitochondrial dysfunction.

Interestingly, it has been shown in mitochondria isolated from the liver that ethanol metabolism sensitizes the mitochondrial permeability transition pore to open, in part, through increased cyclophilin-D activity and increased association of cyclophilin-D with ANT[61]. This increased activity is associated with hyperacetylation of cyclophilin-D. Acetylation of cyclophilin-D is regulated by sirtuin-3, a NAD^+-dependent deacetylase localized in the mitochondrial matrix[62]. The ethanol oxidation-mediated decrease in NAD^+ leads to decreased sirtuin-3 activity and the hyperacetylation of cyclophilin-D. Hyperacetylation of cyclophilin-D results in increased cyclophilin-D activity, increased binding to ANT, and mitochondrial permeability transition pore induction[61]. Thus, it is tempting to speculate that the ethanol oxidation-mediated induction of the mitochondrial permeability transition pore in pancreatic mitochondria is mediated by a similar NAD^+-sirtuin-3-cyclophilin-D axis.

ENDOPLASMIC RETICULUM STRESS AND THE UNFOLDED PROTEIN RESPONSE

Acinar cells are responsible for the production and secretion of large quantities of digestive enzymes. Because of this, in addition to large numbers of mitochondria, acinar cells possess an extensive endoplasmic reticulum network. The endoplasmic reticulum is the major storage site of calcium in the cell, and is the cellular organelle where the proper folding and trafficking of secretory proteins is determined. Endoplasmic reticulum stress resulting from excessive accumulation of proteins, calcium imbalance, oxidative stress, or accumulation of damaged or misfolded protein leads to a response known as the unfolded protein response (UPR)[63].

One hallmark of the UPR is the activation of the IRE1/XBP1 pathway. Inositol-requiring transmembrane kinase/endonuclease 1 (IRE1) splices X-box binding protein 1 (XBP1) messenger RNA, resulting in spliced XBP1. Spliced XBP1 is a transcriptional activator that regulates a number of genes, which encode proteins that act as ER chaperones, are involved in the proper folding of proteins, or are involved in the degradation of damaged or misfolded proteins.

The UPR is activated by pancreatic injury[64]. Additionally, it has been shown that the UPR is activated in acinar cells by long-term ethanol administration to mice[65]. Ethanol mediated UPR was characterized by increased expression of IRE1 and spliced XBP1. Although the UPR was activated, ethanol administration alone did not result in histopathologic changes to the pancreas. In contrast, administration of ethanol to mice with diminished XBP1 expression ($XBP1^{+/-}$ mice) resulted in

acinar vacuolization, cell necrosis, and inflammation[65]. The presence of pathologic changes in the pancreata of XBP1$^{+/-}$ mice led the authors to suggest that the UPR is a protective mechanism in acinar cells during endoplasmic reticulum stress. Exceeding the capacity of the UPR to compensate for endoplasmic reticulum stress results in overt pancreatitis. Thus, if the protective capacity of the UPR is exceeded, this pathway may contribute to the induction and progression of pancreatitis.

THE ROLE OF STELLATE CELLS IN ALCOHOLIC PANCREATITIS

The pancreas, like the liver, has a population of vitamin A storing cells known as stellate cells. Pancreatic stellate cells are periacinar cells located in interacinar and interlobular areas of the pancreas[66,67]. These cells are responsible for the synthesis of extracellular matrix proteins, as well as matrix metalloproteinases (enzymes that degrade extracellular matrix proteins). Thus, it appears that in the healthy organ, pancreatic stellate cells function to maintain the architecture of the pancreas by regulating the deposition and degradation of extracellular matrix components[68]. In response to pancreatic injury, pancreatic stellate cells are activated and transform into myofibroblast-like cells. Activated pancreatic stellate cells synthesize excessive amounts of extracellular matrix proteins. The accumulation of these proteins results in fibrosis. Thus, pancreatic stellate cells are intimately involved in the regulation of both normal and pathologic aspects of the pancreatitis[68,69].

Pancreatic stellate cells of both rat and human origin have the ability to metabolize ethanol through the oxidative pathway[70,71]. Rat pancreatic stellate cells possess alcohol dehydrogenase, the activity of this enzyme is induced when cells are exposed to ethanol concentrations routinely found in the blood of inebriated individuals[70]. Recently, it has also been reported that quiescent pancreatic stellate cells in human beings possess alcohol dehydrogenase activity. Additionally, this activity appeared to be upregulated in pancreatic stellate cells of individuals suffering from chronic pancreatitis and pancreatic cancer[71].

The fact that pancreatic stellate cells possess alcohol dehydrogenase activity may contribute to the development of alcoholic pancreatitis. Pancreatic stellate cells are activated when exposed to concentrations of ethanol detected in the blood of inebriated individuals (10-50 mmol/L)[70,72]. Additionally, pancreatic stellate cells isolated from both rats and human beings are activated by acetaldehyde. Ethanol and acetaldehyde not only activate pancreatic stellate cells, but also elicit responses that may have important biological consequences. Both ethanol and acetaldehyde have been shown to induce the secretion of matrix metalloproteinases in pancreatic stellate cells[73]. Furthermore, treatment of pancreatic stellate cells with ethanol induces the synthesis of interleukin-8 and connective tissue growth factor (CTGF)[72,74]. It has been suggested that these factors act in an autocrine manner to perpetuate the activation of pancreatic stellate cells[13]. This finding may help to explain both the apparent inability of the pancreas to fully recover from injury in the continued presence of ethanol, and the extremely common association between alcohol abuse and chronic pancreatitis.

Although it is well established that pancreatic stellate cells are primarily responsible for the deposition and degradation of components of the extracellular matrix, it appears that acinar cells exposed to ethanol may also contribute to the increase in extracellular matrix deposition. It has been shown that FAEEs can increase the levels of extracellular matrix proteins by inhibiting the acinar cell activity of plasmin and urokinase-type plasminogen activator (uPA) proteins involved in the degradation of the extracellular matrix components[75].

THE ROLE OF THE INFLAMMATORY RESPONSE

Inflammation mediated by cytokines, chemokines, and adhesion molecules is involved in the development of pancreatitis[1,76,77]. Interestingly, it appears that ethanol and its metabolites have a differential effect on the expression of molecules that regulate the inflammatory response. It has been shown that treatment of isolated acini with ethanol or acetaldehyde decreased the activity of two important transcriptional activators involved in the inflammatory response, specifically nuclear factor-κB (NF-κB) and activator protein 1 (AP-1). Conversely, treatment of acini with FAEEs increased the activation of these regulators of the inflammatory response[78].

The activity of NF-κB is also reduced in the pancreata of animals chronically fed ethanol[79]. However, it was demonstrated that induction of pancreatitis in rats chronically administered ethanol resulted in increased NF-κB activity, as well as increases in the mRNA levels of a number of proinflammatory cytokines, including: tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), macrophage inflammatory protein-1 (MIP-1), and monocyte chemotactic protein-1 (MCP-1)[79]. These results led the authors to suggest that the *in vitro* and *in vivo* down-regulation of these factors by ethanol reflected a protective mechanism to prevent the development of alcohol-induced pancreas[78,79].

The role of the inflammatory response in chronic alcoholic pancreatitis has also been investigated[80]. Focusing on the resident mononuclear cells of the pancreas, Deng *et al*[80] demonstrated that chronic ethanol administration reduced the number of these cells present in the pancreas. In agreement with others, they suggested that this reduction likely reflected a general immunologic suppression in the pancreas of ethanol-fed rats, and may explain why animals chronically provided ethanol do not develop chronic pancreatitis in the absence of acute pancreatic damage[80].

Despite this immunologic suppression, when pan-

creatitis was induced by caerulein, the inflammatory response in these animals was enhanced[80]. Furthermore, following repeated caerulein-induced episodes of pancreatitis, it was shown that the expression of both pro-inflammatory cytokines such as TNF-α, MIP-1α, and RANTES (regulated on activation normal T cell expressed and secreted), as well as the anti-inflammatory cytokines tissue growth factor-β (TGF-β) and interleukin-10 (IL-10) was enhanced. The increase in cytokine expression was only observed in rats fed ethanol and subjected to repeated episodes of acute pancreatitis, and was also associated with increased activation of pancreatic stellate cells and fibrosis. These findings led the authors to suggest that ethanol acts not only to sensitize the pancreas to acute pancreatitis, but also aids in the progression of chronic pancreatitis if repeated episodes of acute pancreatitis occur[80].

EFFECTS OF ETHANOL ON PANCREATIC REPAIR

It is generally accepted that fibrosis is an aberrant repair response. It appears that in the presence of ethanol, repair of the damaged pancreas is altered or never fully completed[26,27]. This may help to explain the extremely common association between alcohol abuse and chronic pancreatitis. Because ethanol and acetaldehyde can activate stellate cells, and FAEEs inhibit the degradation of extracellular matrix proteins, it is obvious that ethanol can also influence recovery of the pancreas after damage has occurred[70,72,75].

It has been demonstrated that chronic ethanol administration also delays regeneration of the damaged pancreas[81]. This delay was associated with an ethanol-mediated decrease in the expression of important developmental factors, such as PDX-1 and PTF-1a, as well as impaired activation of the Notch signaling pathway[24]. Normal pancreatic repair requires the dedifferentiation of mature acinar cells followed by their redifferentiation[82]. Thus, ethanol-mediated alterations in the expression of these important developmental factors affect the dedifferentiation/redifferentiation of acinar cells. These alterations may dramatically influence pancreatic repair.

As mentioned above, there is a close association between alcohol abuse and chronic pancreatitis. In fact, in developed countries, alcohol abuse is associated with over 70% of the reported cases[83]. Importantly, individuals suffering from chronic pancreatitis have a 20-fold greater likelihood of developing pancreatic cancer[84], a disease with a dismal prognosis. It is thought that changes that occur in the pancreas during chronic injury are associated with, or predispose the organ to, the initiation of pancreatic neoplasia. Because one of the seminal characteristics of chronic pancreatitis is aberrant tissue repair, resulting in fibrotic scarring, and ethanol consumption alters pancreatic repair, ethanol may have an indirect role in the initiation of pancreatic cancer. Thus, the effects of ethanol on repair of the damaged pancreas may be a contributing factor in pancreatic cancer, as well as alcoholic pancreatitis.

CONCLUSION

Despite the dramatic expansion of our understanding of pancreatitis in general, and how ethanol and its metabolites affect pancreatic cells, we still have not defined the mechanism of alcoholic pancreatitis. Instead, it is evident that ethanol has a plethora of toxic affects on pancreatic cells. Because all of these effects occur simultaneously, it is likely that the cumulative effects of ethanol sensitize the pancreas to damage, and that "alcoholic pancreatitis" is a multifactorial disease. Paradoxically, despite the demonstration that ethanol has numerous toxic effects on the pancreas, data from demographic studies and preclinical animal models has firmly established that ethanol itself does not cause pancreatitis. Because ethanol does not cause pancreatitis, but only sensitizes the pancreas to disease, it appears that the pancreas has developed protective mechanisms that can partially compensate for ethanol-induced cellular damage. Some of these protective mechanisms have been identified. It is likely that additional compensatory mechanisms exist. Further defining the mechanisms of ethanol-induced pancreatic injury may help define these protective mechanisms. It is hoped that this strategy will lead to the development of therapeutic targets that will prevent or reduce the severity of alcoholic pancreatitis.

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TOPIC HIGHLIGHT

WJGP 5th Anniversary Special Issues (3): Pancreatitis

Early phase of acute pancreatitis: Assessment and management

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Abstract

Acute pancreatitis (AP) is a potentially life-threatening disease with a wide spectrum of severity. The overall mortality of AP is approximately 5%. According to the revised Atlanta classification system, AP can be classified as mild, moderate, or severe. Severe AP often takes a clinical course with two phases, an early and a late phase, which should both be considered separately. In this review article, we first discuss general aspects of AP, including incidence, pathophysiology, etiology, and grading of severity, then focus on the assessment of patients with suspected AP, including diagnosis and risk stratification, followed by the management of AP during the early phase, with special emphasis on fluid therapy, pain management, nutrition, and antibiotic prophylaxis.

Key words: Acute pancreatitis; Incidence; Pathophysiology; Etiology; Severity; Risk stratification; Fluid therapy; Pain management; Nutrition; Antibiotic prophylaxis

Core tip: Acute pancreatitis is a frequent and potentially life-threatening disease. Therapy is currently mostly symptomatic with fluid resuscitation, pain management, and early oral feeding. Vigorous fluid resuscitation remains a cornerstone of early management of acute pancreatitis. Cross-sectional imaging during the early phase of evaluation has not been associated with improvement in outcome. There is no role for prophylactic antibiotics in the management of the early phase of acute pancreatitis (AP). Enteral nutrition in AP can reduce mortality, systemic infections, and multiorgan dysfunction compared to parenteral nutrition. Immediate endoscopic retrograde cholangiography is indicated only in patients with biliary pancreatitis with common bile duct obstruction and cholangitis.

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INTRODUCTION

Acute pancreatitis (AP) is a potentially life-threatening disease with a wide spectrum of severity. The reported incidence of acute pancreatitis differs depending on geographic location and ranges from 14.7/100000 person years in the Netherlands to 45.1/100000 person years in Japan[1,2]. However, most studies show an incidence between 30 and 45/100000 person years[2-7]. Many studies report an increase in incidence over the last few decades[2,3,8], however, it is a matter of debate whether this

represents a real increase in incidence due to increasing biliary AP in an increasingly obese population or whether this rise in incidence is due to improved diagnostic capabilities, a higher level of suspicion of this disease, or an overestimation of retrospective studies using administrative diagnostic codes[9-11]. In 2009, AP was the most common principal gastrointestinal diagnosis at discharge in the Unite States with estimated inpatient costs of $2.6 billion per year. Furthermore, it was the 14th most common cause of death with a crude rate of 1.0 per 100000 inhabitants[12]. The overall mortality of AP is about 5% and can reach up to 20%-30% in patients with severe AP and infected necrosis[13,14]. While there seems to be an increase in incidence, several studies have reported a decrease in mortality. Again, this could be a real decrease due to an earlier diagnosis and better therapeutic options or it may also be due to an improved sensitivity of diagnostic modalities, leading to an increase in the diagnosis of mild forms of pancreatitis[15].

In this review article, we first discuss general aspects of AP, including pathophysiology, etiology, and grading of severity, then focus on the assessment of patients with suspected AP, including diagnosis and risk stratification, followed by the management of AP during the early phase with special emphasis on fluid therapy, pain management, nutrition, and antibiotic prophylaxis.

PATHOPHYSIOLOGY

The pathophysiology of AP with multi organ failure (MOF) is poorly understood. Researchers have long hypothesized that AP results from premature activation of digestive enzymes within the pancreas, a process referred to as autodigestion. Indeed, inherited mutations in genes encoding for digestive enzymes have been found in patients with a hereditary form of pancreatitis[16]. However, affected patients develop chronic, rather than acute pancreatitis. Therefore, in recent years, a novel concept has evolved, suggesting that systemic complications during AP result from uncontrolled activation of the inflammatory cascade. As indicated above, severe AP is associated with a significant mortality. Thus, early identification of severe forms of AP is crucial for outcome. In an attempt to identify surrogate parameters as predictors for severe AP, several association studies linking cytokines and chemokines with AP severity have been conducted[17]. Among these, serum levels of interleukin (IL)-6 and the IL-6-dependent acute phase protein, C-reactive protein (CRP) were identified as the most reliable predictors for severe AP[18,19]. Recent results from basic research have established that IL-6 or CRP are not only relevant markers to predict the severity of AP, but that the cytokine IL-6 also has a substantial pathophysiological impact on the course of the disease[19]. While excessive stimulation of the inflammatory cascade [hyper-inflammatory state, systemic inflammatory response syndrome (SIRS)] accounts for early systemic complications, paralysis of the inflammatory response, also termed compensatory anti-inflammatory response syndrome (CARS), contributes to local complications and sepsis associated with the late phase of the disease. Although these definitions are largely non-specific, they are undeniably useful in the clinical and research setting. Among the agents contributing to this anti-inflammatory response, IL-10 may be of importance. In fact, the protective role of IL-10 in experimental studies in animal models has been well documented[20]. Thus, the hypo-inflammatory status of CARS might facilitate superinfections that lead to extensive necrosis and/or septic complications. This interplay of these two contrasting phenomena requires an individualized therapeutic approach[20-22].

ETIOLOGY

The identification of the etiology of AP is crucial for the management during the early phase of the disease and also for the prevention of recurrence of AP. Although there is no specific therapy for AP, the causing factor, *e.g.*, choledocholithiasis in biliary AP, must be investigated and eliminated if identified. The most common causes of AP are gallstones and prolonged heavy use of alcohol, which together account for about 60%-80% of all cases. The incidence of biliary etiology differs considerably between different geographic regions. For example, there is a clear predominance for biliary AP over alcoholic AP in Greece (71.4% *vs* 6.0%) whereas the opposite is the case for Finland (6.3% *vs* 79.3%)[23,24]. The regional differences in frequency of biliary and alcoholic etiology are shown in Figure 1[6,7,23-31].

Other causes of AP include ERCP (0.4% to 11%[32,33]), idiosyncratic reactions to drugs (0.1% to 2%)[34], hypertriglyceridemia (1.1%-3.8%)[6,23,35], anatomic alterations[36], genetic predispositions[37], and other rare causes[38,39]. Despite a thorough clinical workup, 10%-25% of all cases remain idiopathic[6,11,23,33].

NATURAL COURSE OF ACUTE PANCREATITIS

The severity of AP can be subclinical, mild without organ dysfunction, or can be severe. Patients with mild disease often improve spontaneously and heal within a few days. However, patients with severe disease may develop life-threatening local and/or systemic complications. According to the revised Atlanta classification system, AP can be classified as mild, moderate, or severe[40]. However, it is important to remember that AP is a rapidly evolving, dynamic condition in which the severity may change rapidly during the course of the disease[40]. Severe AP often takes a clinical course with two phases, an early and a late one, which should both be considered separately[40].

The early phase, which usually lasts for about one week, is characterized by a complex inflammatory reaction. The course of AP starts with a systemic proinflammatory phase systemic inflammatory response syndrome

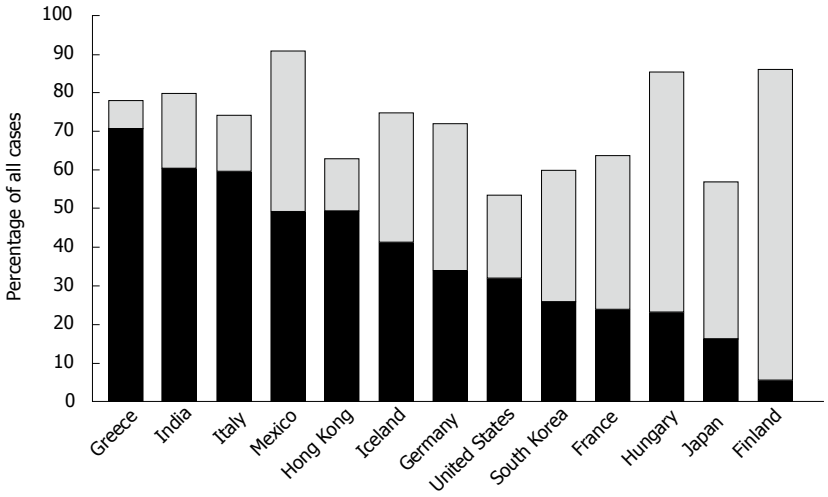

Figure 1 Regional differences in frequency of biliary (black) and alcoholic (gray) etiology of acute pancreatitis.

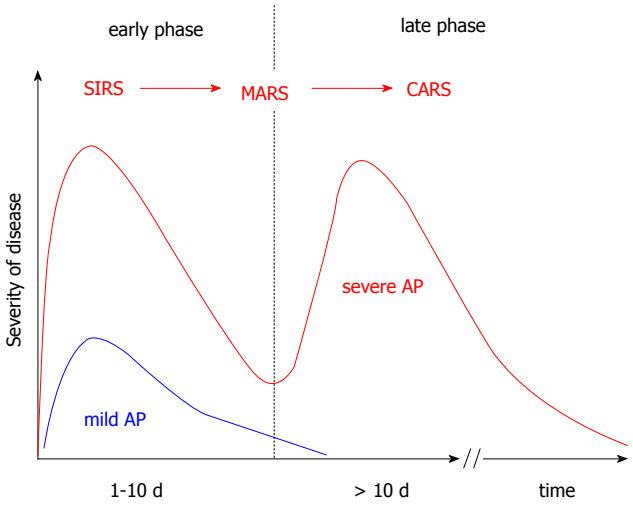

Figure 2 Two-phase course of severe acute pancreatitis. CARS: Compensatory anti-inflammatory response syndrome; MARS: Mixed anti-inflammatory response syndrome; SIRS: Systemic inflammatory response syndrome.

(SIRS), followed by a mixed inflammatory response syndrome mixed antagonist response syndrome (MARS), and finally leads to a phase with a suppressed inflammatory response compensatory anti-inflammatory response syndrome (CARS)[41-43]. In the phase of CARS, the immune system is downregulated and the chance of an infection of pancreatic and peripancreatic necrotic tissue rises. This is likely the reason why infections usually do not occur earlier than at the end of the first week[44]. During the stage of CARS pathogens can migrate unopposed from the intestinal lumen into necrotic tissue in and around the damaged pancreas. At that point, the clinical course of AP moves towards the second phase, including SIRS, sepsis, local and systemic complications, persistent organ failure, and possibly death. The model of the two-phase course is shown in Figure 2.

Efforts must be made to predict the severity of the disease as early as possible in order to know whether a patient diagnosed with AP can be treated as an outpatient, has to be admitted to a regular ward, to an intermediate care facility, or even to the intensive care unit. While it is generally recognized how important the prediction of severity of the disease is for the management of the individual patient, it is also recognized that such prediction is very difficult. Underestimation of the severity could be harmful for the patient, while overestimation could lead to unnecessary costs and a waste of resources. Therefore, the assessment and prediction of the severity is crucial for the management of the disease. A lot of research has been done over the last few decades trying to identify new tools to accurately predict the severity of pancreatitis, yet no gold standard for such prediction of the course of AP has been identified. An ideal predictor should be fast and easy to obtain, widely available, economical, and associated with a high sensitivity and specificity. Even though there are several clinical scores with a high sensitivity, specificity, positive, and/or negative predictive value, many of them are complicated to asses or can predict severity only after 48 h of admission to the hospital, which effectively means more than 72 h after the onset of disease[45]. This might be too late, as early aggressive fluid resuscitation is a cornerstone of AP therapy.

ASSESSMENT

Diagnosis

The diagnosis of AP can be made if ⩾ 2 of the following three criteria are fulfilled: (1) abdominal pain characteristic of acute pancreatitis; (2) elevation of serum lipase or amylase activity > 3-fold of the upper limit of the reference interval; and (3) characteristic signs of pancreatitis on computed tomography (CT) imaging.

The first step in the diagnosis of AP should be a thorough clinical history. The pain caused by AP is typically dull, located in the epigastrium, may radiate into the back, and is usually severe, leading to hospital admission and often necessitating opioid therapy[45,46]. Furthermore, AP often causes nausea and vomiting. Known cholecystolithiasis and/or colics, alcohol excess within 48 h before the onset of pain, new medications, and the character of the pain should be evaluated. The second step of pancreatitis diagnosis is based on clinical

Table 1 Prognostic criteria of Ranson

On admission	After 48 h
Age > 55 yr	Hematocrit fall > 10%
White blood cell count > 16000/mL	BUN increase > 1.8 mmol/L
Blood glucose concentration > 11.1 mmol/L	Serum calcium < 2 mmol/L
LDH > 350 IU/L	PaO_2 < 60 mmHg
ASAT > 250 IU/L	Base deficit > 4 mmol/L
	Fluid sequestration > 6 L

ASAT: Aspartate aminotransferase; BUN: Blood urea nitrogen; LDH: Lactate dehydrogenase; PaO_2: Partial pressure of arterial oxygen.

chemistry. The measurement of serum lipase activity is generally thought to be more sensitive and specific than that of serum amylase activity and there is no additional value in simultaneous measurement of serum lipase and amylase activities[14,47]. Also, the degree of the elevation of serum pancreatic enzyme activities does not correlate with the severity of the disease, although, some studies would suggest such a correlation between serum enzyme activity and severity[6,45]. Only in patients with characteristic epigastric pain, but serum enzyme activities below 3-fold of the upper limit of the reference interval, a CT scan should be considered to rule out other differential diagnoses or to confirm AP. Apart from that, a CT in the early phase of AP is not recommended by current practice guidelines[14,48,49].

Risk stratification

Risk factors: Obesity favors the development of local and systemic complications in patients with AP[50]. Since assessment for obesity is simple and free it should be assessed in every patient. The same applies for age, as patients 55 years or older are at increased risk for severe disease[14].

Scoring systems: Several single parameters and more or less complex scoring systems for the prediction of the severity of AP have been developed and clinically evaluated and all of them have been shown to be associated with advantages and disadvantages. The HAPScore (harmless acute pancreatitis score) was developed to identify patients with mild AP who can be treated as outpatients. Patients without rebound tenderness and/or guarding, a normal hematocrit, and a normal serum creatinine concentration have a high probability (positive predictive value: 98%-98.7%) to have a harmless course of the disease[51,52].

One of the oldest and probably best known and heavily used scores to predict a severe course of pancreatitis was developed in the early 70ties by John Ranson and colleagues[53]. The Ranson score is based on the presence or absence of simple parameters and is assessed differently at the time of admission (5 parameters; possible scores: 0-5) and 48 h later (6 parameters; possible scores: 0-6; Table 1).

Although a score ⩾ 3 has a high sensitivity and specificity regarding a severe course of pancreatitis (83.9% and 78.0%, respectively) and a negative predictive value of 94.5%, the severity can be predicted no earlier than 48 h after admission[25,54]. A modification of the Ranson score by Clemens Imrie and colleagues (Imrie score or Glasgow score) was first reported in 1978 and is still widely used and has a similar accuracy as the Ranson score[25,55].

Table 2 Bedside index of severity in acute pancreatitis score and observed mortality by bedside index of severity in acute pancreatitis score score

BUN > 8.9 mmol/L	
Impaired mental status (Glasgow coma scale < 15)	
SIRS, defined by the presence of two or more	
Temperature	< 36 ℃ or > 38 ℃ (< 96.8 °F or > 100.4 °F)
Heart rate	> 90 per minute
Respiratory rate	> 20 per minute or $PaCO_2$ < 32 mmHg
White blood cell count	< 4000/mL or > 12000/mL or > 10% immature neutrophils
Age > 60 yr	
Pleural effusion	
BISAP score	Mortality (%)
0	0.1-0.2
1	0.5-0.7
2	1.9-2.1
3	5.3-8.3
4	12.7-19.3
5	22.5-26.7

BUN: Blood urea nitrogen; $PaCO_2$: Partial pressure of arterial carbon dioxide; SIRS: Systemic inflammatory response syndrome.

Currently, the score with the highest sensitivity regarding prediction of a severe course is the Acute Physiology And Chronic Health Evaluation (APACHE) Ⅱ score[14,56]. Originally developed to predict mortality in intensive care patients, a value ⩾ 8 of the APACHE Ⅱ score predicts a severe course of AP with a sensitivity of 65%-83%, specificity of 77%-91%, positive predictive value (PPV) of 23%-69%, and negative predictive value (NPV) of 86%-99%[54,57]. However, the determination of an APACHE Ⅱ score in a clinical patient is complex and time-consuming as it utilizes more than 15 parameters, which limits the clinical value of this score.

A score that was developed and validated more recently in almost 18000 patients, is the BISAP (Bedside Index of Severity in Acute Pancreatitis) score[58]. The main advantage of the BISAP score is its simplicity. One point each is given for blood urea nitrogen (BUN) > 8.9 mmol/L, impaired mental status (Glasgow Coma Scale < 15), presence of SIRS, age > 60 years, and pleural effusion (Table 2). A score ⩾ 3 is predictive for a severe course (observed mortality of > 5%; Table 2) with a sensitivity of 83% and a PPV of 76.9%[58-60]. One disadvantage of the BISAP score is, that this score cannot easily distinguish patients with transient and persistent organ failure and therefore may overestimate severity and preclude differentiation between moderate and severe AP.

In summary, there is currently no ideal predictor of severity of AP. All prognostic factors and scores show a

Table 3 Balthazar score

Grade A	Normal pancreas
Grade B	Focal or diffuse enlargement of the pancreas
Grade C	Pancreatic changes associated with peripancreatic inflammation
Grade D	Single fluid collection
Grade E	Two or more fluid collections and/or presence of gas within the pancreas or within peripancreatic inflammation

Table 4 Computed tomography severity index

Extent of necrosis	Points
Absence of necrosis	0
< 30% necrosis	2
30%-50% necrosis	4
> 50% necrosis	6
Balthazar score	
A	0
B	1
C	2
D	3
E	4

Maximum score 10 points.

good NPV, but suffer from a low PPV. Thus, the main value of severity assessment is to exclude a large number of patients with a low risk of mortality[57].

In addition to the laboratory/clinical scoring systems described above there are scoring systems based on imaging results to assess and predict the severity of AP. A CT scan for diagnostic purposes and severity assessment has been-and probably still is-standard practice in many centers[61]. The Balthazar score, developed in 1985, categorizes patients with AP into 5 groups (A-E) according to pancreatic and peripancreatic changes diagnosed by CT (Table 3)[62]. In 1990, Balthazar *et al*[63] modified this score, including assessment of the extent of pancreatic necrosis and named this score Computed Tomography Severity Index (CTSI) (Table 4). The CTSI is probably the most frequently used imaging score to assess severity in patients with AP and a score ⩾ 4 has a negative predictive value of 94%-97% and a positive predictive value 53%-69% regarding the clinical severity of disease[61,64].

In addition to the Balthazar score and the CTSI, several other scores, *e.g.*, pancreatic size index (PSI), mesenteric edema and peritoneal fluid (MOP) score, extrapancreatic (EP) score, extrapancreatic inflammation on CT (EPIC) score, modified CTSI (MCTSI), and MR severity index (MRSI) have been developed and evaluated[61,65]. However, none of these imaging scores were shown to be superior to clinical scoring systems. Thus, a CT on admission to predict severity of AP cannot be recommended at the current time[61].

In addition to laboratory/clinical and imaging scoring systems, single parameters have been evaluated to assess and predict severity.

A lot of research has been done evaluating hematocrit as an indicator for hemoconcentration. The first prospective cohort study showed a high NPV for a hematocrit ⩾ 44 % (93% on admission and 97% 24 h later) but a poor PPV (26% and 27%, respectively) regarding organ failure in AP[66]. Similar results were obtained by several other studies focusing on the usefulness of hematocrit to predict a severe course of AP, organ failure, pancreatic necrosis, or death[67,68]. Due to its high negative predictive value, its low cost, and the ease of measurement, the hematocrit has value in predicting a non-severe course of AP.

The disruption of water balance can lead to hypoperfusion and a disturbance of pancreatic microcirculation[69], which in turn correlates with the severity of AP[70,71]. Understanding the water balance and the resulting changes in laboratory tests can help to predict severity and outcome of AP. In addition to hematocrit, other parameters, that mirror intravascular volume depletion, can also be helpful.

Serum creatinine has been identified as a predictor for pancreatic necrosis. Also, more recently, an estimated glomerular filtration rate (GFR) < 90 mL/min per 1.73 m^2 on admission has been shown to predict pancreatic necrosis with a sensitivity, specificity, PPV, and NPV of 78.1%, 71%, 64%, and 83%, respectively[72,73]. While only one study has described GFR as a predictor of severity, BUN has been evaluated for many years and has been shown to be a good predictor for severity in AP in several large studies. A rise in BUN > 1.8 mmol/L after 48 h had already been included in the Ranson score 40 some years ago, is one of the 4 parameters used in the BISAP score, and has also been shown to have a high predictive value as a single parameter[74,75].

Besides parameters focusing on water balance and microcirculation, laboratory parameters suggesting the presence of an inflammatory process have been used as a predictor of severity. The most intensively studied parameter is CRP. In one study, a serum CRP concentration of 150 mg/L or greater predicted severe AP at 36 h after admission with a sensitivity, specificity, PPV, and NPV of 86%, 87%, 75%, and 93%, respectively[76]. However, the prediction of severity was only possible more than 24 h after admission, which, on average, is about 50 h after the onset of pain[45]. Also, several other studies showed a high predictive value of CRP during the course of AP in regards to severity, but a very low predictive value on admission[77,78].

Procalcitonin appears to be a valuable tool to discriminate between sterile and infected necrosis within the first days of AP[79,80]. However, data on the ability to predict the course of AP are not consistent. On one hand, a multicenter study from the United Kingdom found a significant difference of procalcitonin concentrations measured within 48 h of the onset of symptoms in patients with mild and severe AP and showed an accuracy of 94% in predicting death[81]. In a study from Slovakia, the PPV for predicting a fatal outcome reached 75% when a cut-off value of 5 ng/mL was used[82]. A

third study evaluating procalcitonin showed an accuracy of 76% and a PPV of 75% for predicting a severe course of pancreatitis[83]. On the other hand, two studies reported that procalcitonin is not useful in predicting the severity of AP upon admission[79,84]. However, the time point for determination of procalcitonin concentrations, the assays used, and the cut-off values applied were different for all studies. Finally, measurement of procalcitonin is not widely available and is expensive.

A blood glucose concentration < 6.9 mmol/L on admission has a high negative predictive value (92%) for pancreatic necrosis and also can serve as a predictor for severity[85,86]. Blood glucose is easy, fast, and inexpensive to determine and widely available and therefore should be included in the risk stratification.

In summary, there is no single marker that can adequately predict the severity of AP, but there are several scoring systems that can be used to assess and predict the severity of AP. However, these scoring systems must be applied at the correct time, the correct place, and in the correct patient. Also, it is important to observe patients carefully and reassess severity frequently as the disease course can change rapidly at any given time.

MANAGEMENT

Patients diagnosed with mild AP (according to the HAPScore) and no other risk factors can be treated as outpatients. In contrast, patients with any of the above-mentioned risk factors should be considered for admission to the hospital for close monitoring and timely reassessment of disease severity. In contrast, patients with a Ranson score ⩾ 3, a BISAP score ⩾ 3, an APACHE-II score ⩾ 8, or patients with apparent organ failure should be transferred to an advanced medical care ward or facility.

Therapy

Fluid therapy: Despite a lot of research, there is no pharmacological treatment of AP[87]. Thus, fluid resuscitation, analgesia, supportive care, and management of the local and systemic complications are the key elements of the management of patients with acute pancreatitis. One of the most important components of therapy of AP is early intravenous fluid resuscitation[88]. In fact, the decrease in mortality observed over the last decade might be due to the prevention of pancreatic necrosis by maintenance of microcirculation due to more aggressive fluid resuscitation[89]. Two studies have shown a decrease in mortality by early and aggressive fluid resuscitation[90,91]. However, data on the amount of fluid needed to prevent necrosis or to improve outcome are contradictory and the volume must be adjusted to the patient's age, weight, and pre-existing renal and/or cardiac conditions[92]. The importance of starting fluid resuscitation as early as possible and in fact already in the emergency room was shown by two retrospective studies[90,91]. However, the optimal type of fluid is still a matter of debate. Studies comparing isotonic saline and lactated Ringer's solution and crystalloid *vs* colloid solutions, respectively, showed no differences between both groups regarding clinical outcome as determined by the frequency of pancreatic necrosis, length of hospital stay, or mortality[93,94]. Also, the optimal therapeutic goal of fluid resuscitation is not yet clear. A goal-directed fluid resuscitation algorithm based on changes in BUN measurements, as a mirror of renal function, showed no improvement in outcome in patients with AP[93]. Nonetheless, blood pressure, respiratory function, urine output, and-where appropriate-intraabdominal pressure should be closely monitored. One study showed a less severe course of post-ERCP pancreatitis when patients were treated according to a fluid resuscitation protocol based on vital signs and hematocrit[95]. While questions on the type of fluid, the optimal rate of administration, and the therapeutic goal to reach remain unanswered[96], the time-point appears to be very important - the earlier, the better[90,91].

Causative therapy: Elimination of any potential risk factor is another important approach to AP therapy. In case of suspected alcohol- or drug-induced AP, the intake of the causing agent must be stopped immediately. In case of biliary AP, the indication to perform an endoscopic retrograde cholangiography (ERC) and removal of stones within the bile duct depends on the degree of obstruction of the common bile duct and the presence of cholangitis. Biliary pancreatitis and cholangitis are clear indications for ERC and ERC should be performed as early as possible[49,97,98]. Immediate ERC is indicated in patients with biliary pancreatitis with common bile duct obstruction and cholangitis, arguable in patients with predicted severe pancreatitis but without cholangitis, and not indicated in predicted mild pancreatitis without cholangitis[49].

After biliary pancreatitis, cholecystectomy is recommended within the same hospital stay for mild pancreatitis or after an interval of 6 wk following an episode of severe pancreatitis[49].

Pain management: Given that most patients with AP suffer from severe pain, adequate analgesia is very important. In mild cases, non-opioid drugs might be satisfying, but in many cases, especially severe AP, parenterally administered narcotic agents are warranted and most patients will require the use of opioids to control the pain[99,100]. In contrast to historical reports, there is no evidence or a recommendation for restrictions on the type of pain medications being used[14].

Nutrition: For many years, resting the pancreas by giving the patient nothing per os was an important part of therapy. Nowadays, there is wide agreement that total oral abstinence from food combined with total parenteral nutrition is not beneficial to patients with severe AP, but may in fact be detrimental. A recent meta-analysis

showed a statistically significant association of early enteral nutrition and reductions in systemic infections, pancreatic infections, length of hospital stay, and mortality[101]. Also, in patients with severe AP, enteral nutrition was significantly superior to total parenteral nutrition regarding mortality, infectious complications, and organ failure[102]. Gut barrier function is compromised in patients with acute pancreatitis, likely leading to bacterial translocation and potentially causing infected necrosis or even sepsis[103,104]. Because enteral feeding stabilizes gut barrier function, thereby reducing bacterial translocation, it is important early during the course of AP[14,105].

Therefore, whenever possible, *i.e.*, when dissipating pain allows the patient to eat and infectious parameters do not continue to rise, oral food intake should be initiated as early as possible[49]. If oral food intake is not possible and the patient needs nutritional support, enteral tube feeding is preferred over total parenteral nutrition. However, the composition of an optimal diet has not yet been evaluated.

Antibiotic prophylaxis: There also has been a change regarding prophylactic antibiotic therapy in patients with AP. While in the 90ties, prophylactic antibiotics where thought to improve the outcome in patients with AP, there is no emerging evidence that prophylactic antibiotics reduce infectious complications or mortality[106-108]. Today, there is no clear evidence that supports antibiotic prophylaxis as a routine treatment in patients with severe AP[109-111]. Prophylactic antibiotics may reduce pancreatic infection in special subgroups of patients, but further well-designed and adequately-powered studies are needed to definitively answer the clinical usefulness of antibiotic prophylaxis in these patients[108]. Therefore, antibiotic prophylaxis is currently not recommended by international guidelines for the treatment of acute pancreatitis[14,49].

CONCLUSION

Acute pancreatitis is a frequent and potentially life-threatening disease. Numerous clinical prognostic scoring systems have been developed, and yet tools to discriminate between mild, moderate, and severe AP early during the course of the disease are not well advanced. Therapy is currently mostly symptomatic with fluid resuscitation, pain management, and early oral feeding. However, most of these therapeutic approaches are not well-defined. Vigorous fluid resuscitation remains a cornerstone of early management of acute pancreatitis. Cross-sectional imaging during the early phase of evaluation has not been associated with improvement in outcome. There is no role for prophylactic antibiotics in the management of the early phase of AP. Enteral nutrition in AP can reduce mortality, systemic infections, and multiorgan dysfunction compared to parenteral nutrition. Immediate ERC is indicated only in patients with biliary pancreatitis with common bile duct obstruction and cholangitis. These developments have contributed to an improved outcome for patients with acute pancreatitis, but further studies are still required to tackle the high mortality in this disease.

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TOPIC HIGHLIGHT

WJGP 5th Anniversary Special Issues (3): Pancreatitis

Potential role of NADPH oxidase in pathogenesis of pancreatitis

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Abstract

Studies have demonstrated that reactive oxygen species (ROS) are closely related to inflammatory disorders. Nicotinamide adenine dinucleotide phosphate oxidase (NOX), originally found in phagocytes, is the main source of ROS in nonphagocytic cells. Besides directly producing the detrimental highly reactive ROS to act on biomolecules (lipids, proteins, and nucleic acids), NOX can also activate multiple signal transduction pathways, which regulate cell growth, proliferation, differentiation and apoptosis by producing ROS. Recently, research on pancreatic NOX is no longer limited to inflammatory cells, but extends to the aspect of pancreatic acinar cells and pancreatic stellate cells, which are considered to be potentially associated with pancreatitis. In this review, we summarize the literature on NOX protein structure, activation, function and its role in the pathogenesis of pancreatitis.

Key words: Nicotinamide adenine dinucleotide phosphate oxidase; Reactive oxygen species; Pancreatitis; Pancreatic acinar cells; Pancreatic stellate cells

Core tip: Besides directly producing the detrimental highly reactive reactive oxygen species (ROS) to act on biomolecules, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase can also activate multiple signal transduction pathways, which regulate cell growth, proliferation, differentiation and apoptosis by producing ROS. Recently, research on pancreatic NADPH oxidase is no longer limited to inflammatory cells, but extends to the aspect of pancreatic acinar cells and pancreatic stellate cells, which are considered to be potentially associated with pancreatitis.

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INTRODUCTION

Studies have demonstrated that reactive oxygen species (ROS) are involved in the pathogenesis of pancreatitis[1]. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX), a transmembrane flavoprotein enzyme, uses NADPH as an electron donor to catalyze the univalent reduction of oxygen, resulting in the production of superoxide free radical, which might be a source of oxi-

dants in injured pancreas[1]. NOX is mainly distributed in the phagocytic cell membrane with cytochrome C and *flavin adenine dinucleotide* groups, which can produce ROS, scavenging pathogenic microorganisms such as bacteria[2]. ROS, being generated by NOX, also participate in intracellular signaling processes in the pancreas. Recently, research on NOX is no longer limited to inflammatory cells, but extends to the aspect of pancreatic acinar cells and pancreatic stellate cells (PSCs) in pancreatitis patients[2]. The function of NOX, which is involved in the pathogenesis of inflammation in pancreatic acinar cells and PSCs, has become the hotspot of research. Non-phagocytic NOX derived ROS function as a messenger molecule to participate in the modulation of cell differentiation, proliferation and apoptosis in the pancreas. In this review, we summarize the literature on NOX protein structure, activation, function and its role in the pathogenesis of pancreatitis.

STRUCTURE, LOCATION AND FUNCTION OF NOX IN THE PANCREAS

NOX is a multicomponent enzyme consisting of five different subunits, including the subunits $p22^{phox}$ and $gp91^{phox}$ (also known as NOX2) located in the membrane, together with the cytosolic subunits $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$. The participation of Rac would elicit full oxidase activity[3-5]. Relative to $gp91^{phox}$ (the catalytic subunit of NOX), $p22^{phox}$, $p47^{phox}$, $p40^{phox}$ and $p67^{phox}$ are regulatory subunits. $Gp91^{phox}$ in different types of cells has other six homologues, termed NOX1, NOX3, NOX4, NOX5, DUOX1 and DUOX2, which constitute the NOX family proteins[6-8]. NOX is an enzyme which was initially discovered in phagocytes[4,5]. NOX in neutrophils is composed of constitutive subunits ($p22^{phox}$ and $gp91^{phox}$) positioned in membrane and regulatory subunits ($p47^{phox}$ and $p67^{phox}$, and possibly $p40^{phox}$) stationed in the cytosol[9]. In recent years, NOX has been discovered in several nonphagocytic cells such as fibroblasts[10], vascular smooth muscle cells[11] and hepatic stellate cells[12]. More recently, it has been found that NOX was present in pancreatic β cells[13,14], pancreatic acinar cells[15-18] and PSCs[19,20]. The main intrinsic components of NOX comprising the NOX2 isoform are present in human pancreatic islets[14]. Cytosolic subunits $p47^{phox}$ and $p67^{phox}$ as well as membrane-bound subunits $p22^{phox}$ and NOX1 are constitutively expressed in pancreatic acinar AR42J cells[16,21,22]. The key subunits of NOX including $p22^{phox}$, $p47^{phox}$, NOX activator 1 (a homologue of $p67^{phox}$), NOX1, NOX4, and NOX2 ($gp91^{phox}$) are expressed in PSCs[19,20]. The activation of non-phagocytic NOX is similar to that in neutrophils[23]. Upon activation of NOX, p47 translocates to the membrane and then recruits p67 to interact with the p22 subunit, thus facilitating NADPH-dependent formation of superoxide (O^{2-}), which increases the production of secondary ROS such as hydrogen peroxide (H_2O_2)[21]. Non-phagocytic NOX derived ROS function as a messenger molecule to participate in the modulation of cell differentiation, proliferation and apoptosis[6-8]. NOX protein family can be activated quickly under pathophysiological conditions, leading to high production of ROS, which contributes to oxidative stress and a wide range of diseases.

ACTIVATION AND INHIBITION FACTORS OF NOX IN THE PATHOGENESIS OF PANCREATITIS

Cholecystokinin analogues

Cerulein, an analogue of cholecystokinin (CCK), can stimulate the pancreatic exocrine secretion by binding CCK receptors, causing the autolysis of pancreatic acinar cell[24]. There are two kinds of CCK receptor subtypes, CCK_1 and CCK_2 receptors. CCK_1 receptors regulate pancreatic digestive enzymes, satiety and feeding behavior, while CCK_2 receptors enhance the level of gastric acid, as well as gastrin which has anti-apoptotic effects on pancreatic cells[25]. Experimental pancreatitis induced with high dosages of cerulean, similar to human edematous pancreatitis, is characterized by cytoplasmic vacuolization, formation of edema and acinar cell death as well as elevation in serum levels of digestive enzymes caused by unconventional secretion of digestive enzymes[26]. ROS are involved in the activation of oxidant-sensitive nuclear transcription factor (NF-κB), expression of cytokine, apoptosis and further occurrence of pancreatitis[27]. $P47^{phox}$, $p67^{phox}$, NOX1 and $p22^{phox}$ in pancreatic AR42J cells could produce ROS after cerulein stimulation[21]. Intrapancreatic trypsin is not only activated by high-dose cerulein, but also regulated by neutrophils *via* NADPH oxidase[28]. The mechanism for the activation of NF-κB and expression of cytokines in pancreatic acinar cells stimulated by cerulein may be summarized as the following steps. Cerulein binds to the CCK receptor, a G-protein-coupled receptor, to activate phospholipase C (PLC) and inositol 1,4,5-trisphosphate (IP_3), triggering transient Ca^{2+} release from the endoplasmic reticulum in pancreatic acinar cells. NOX activated by Ca^{2+} produces ROS to activate IκB kinase and then to phosphorylate IκB. Phosphorylated IκB can be ubiquitinated and degraded in a proteasome dependent manner to eliminate the inhibition of NF-κB, a p65/p50 heterodimer in the cytosol. NF-κB then translocates to the nucleus to mediate the expression of cytokines which are involved in the pathogenesis of pancreatitis (Figures 1 and 2)[27].

Renin-angiotensin system

The Renin-angiotensin system (RAS) is generally considered to regulate blood pressure and body fluid homeostasis[29]. The pancreatic RAS activation that is related to the production of ROS might contribute to oxidative stress and tissue injury[30,31]. Angiotensin Ⅱ, an active mediator of RAS, is transformed from angiotensin I by the angiotensin-converting enzyme (ACE)[32]. The effect of angiotensin Ⅱ is regulated by its receptors, including

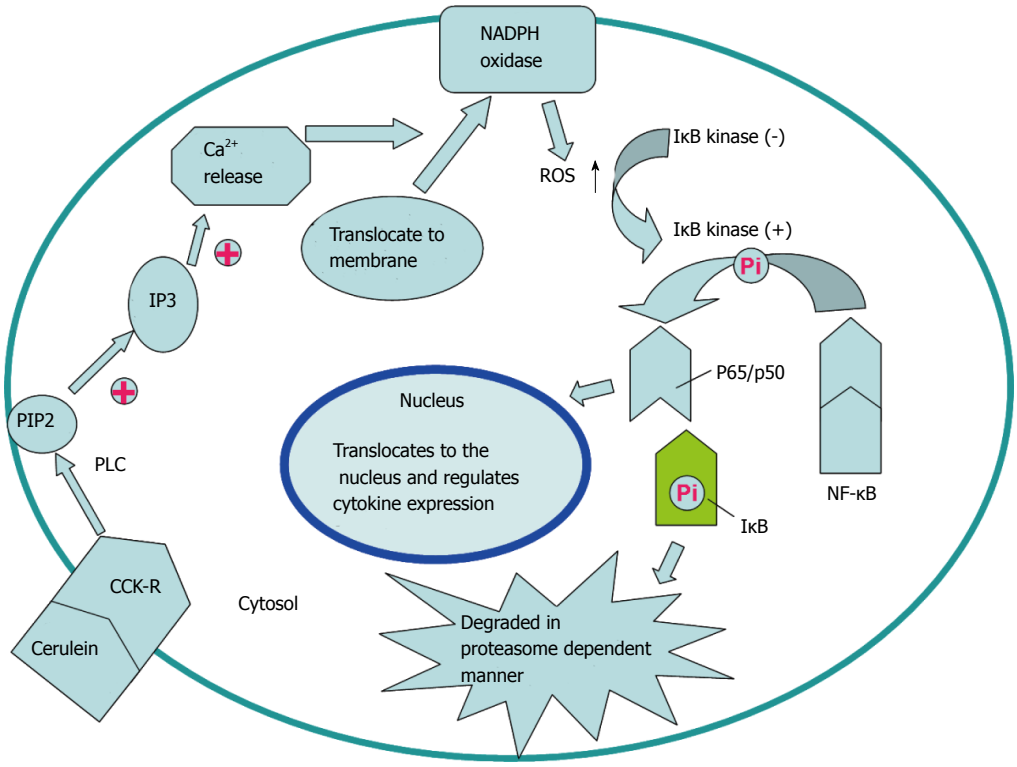

Figure 1 Potential mechanism of nicotinamide adenine dinucleotide phosphate oxidase activation *via* cholecystokinin receptor. Cerulein and cholecystokinin (CCK) receptor binding triggers transient Ca^{2+} release from the endoplasmic reticulum to activate nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which is mediated by PLC and IP3. Reactive oxygen species (ROS) generated by NADPH oxidase activate IκB kinase to phosphorylate IκB in the cytosol. Phosphorylated IκB is ubiquitinated and degraded in a proteasome-dependent manner. NF-κB translocates to the nucleus and regulates expression of cytokines to participate in the pathogenesis of pancreatitis.

angiotensin Ⅱ type 1 receptor (AT_1R) and angiotensin Ⅱ type 2 receptor (AT_2R)[32]. Many reports indicate that interaction of angiotensin Ⅱ with AT_1R promotes superoxide anion production through NOX system[30,31,33,34]. Inhibition of the AT_1R, but not AT_2R, may play a significant role in decreasing the severity of acute pancreatitis. Mechanism of NOX activation by AT_1R and AT_2R might contribute to different effects of AT_1R and AT_2R inhibitors on pancreatic injury induced by cerulein. Activation of pancreatic NOX was associated with oxidative stress which can be indicated by the level of protein oxidation in rats stimulated with cerulein[30,35]. However, further investigations about the potential application of RAS inhibitors including AT_1R in treating acute pancreatitis are needed in the future (Figure 2).

Ethanol and platelet derived growth factor

Alcohol abuse has long been recognized as the most common factor leading to chronic pancreatitis[36]. Activated stellate cells are viewed as vital regulators of chronic alcoholic pancreatitis or fibrosis. Hu *et al*[20] investigated the mechanisms of action of alcohol on PSCs to determine the correlation of NOX system and alcohol with the proliferation of PSCs. The results demonstrated that NOX activity was predominantly located in the cell membrane fraction (95%) compared to the cytosolic fraction (5%) of the stellate cells. platelet derived growth factor (PDGF) could increase NOX activity in a dose- and time-dependent manner. PSC proliferation caused by alcohol is mediated by the activation of PDGF induced NADPH oxidase system. However, ethanol did not show a significant effect on stellate cell DNA synthesis, which provides a new perspective for the mechanism of fibrosis stimulated with alcohol (Figure 2)[20].

Vasoactive intestinal peptide

Previous reports found that vasoactive intestinal peptide (VIP) could decrease the production of cytokines to alleviate experimental acute pancreatitis[37]. VIP could decrease the level of ROS significantly and increase cell viability in acini cells in a dose dependent manner. NOX_1 and NOX_2 markedly increased following treatment with H_2O_2 in pancreatic acini. Besides, H_2O_2 can stimulate the activation of NOX. The production of ROS was affected by VIP *via* NADPH oxidase and the cAMP/PKA pathway because decreased NOX activity by administration of VIP could be abolished by PKA inhibitor H89. Oxidative stress and tissue injury in acini can be decreased by VIP through NOX inhibition (Figure 2)[38].

NOX SIGNAL TRANSDUCTION IN THE PATHOGENESIS OF PANCREATITIS

NOX protein family can be activated quickly under pathophysiological conditions, leading to high produc-

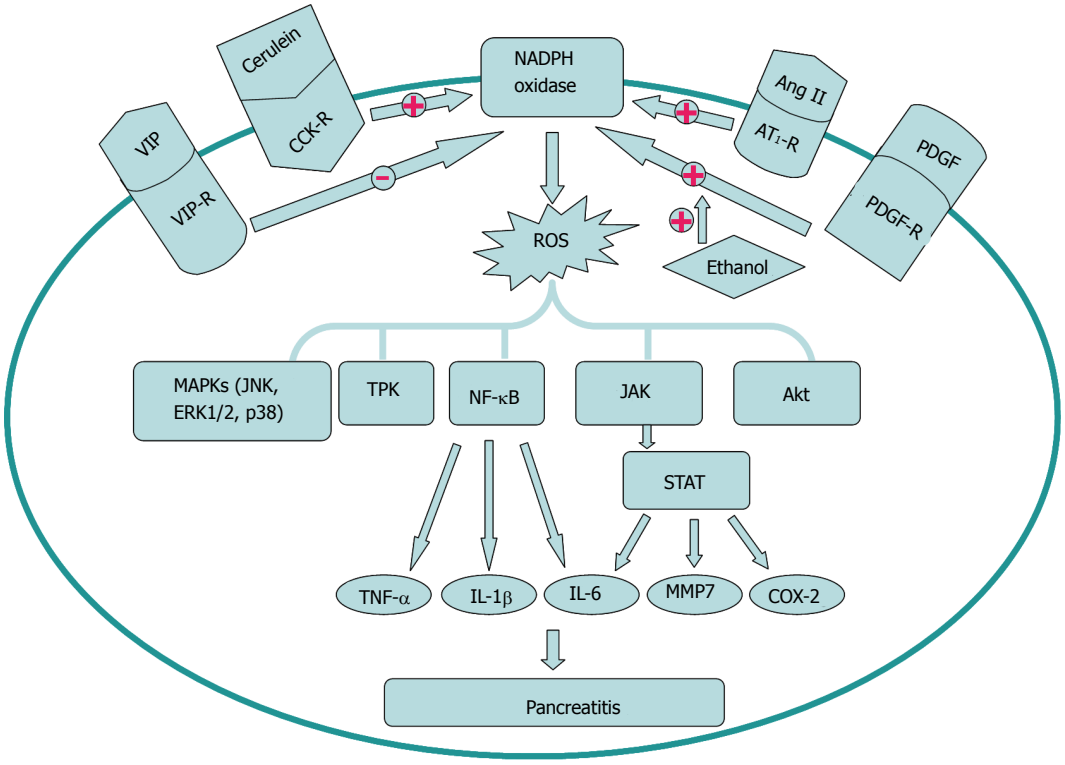

Figure 2 Activation and inhibition factors of nicotinamide adenine dinucleotide phosphate oxidase signal transduction in the pathogenesis of pancreatitis. Cerulein, Ang II and platelet derived growth factor (PDGF) can enhance, while VIP can decrease the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Ethanol can augment the activation of the cell's NADPH oxidase system stimulated by PDGF. The downstream signal molecules including MAPKs, TPK, NF-κB, JAK/STAT and Akt participate in the pathogenesis of pancreatitis. TNF: Tumor necrosis factor; IL: Interleukin; TPK: Tyrosine protein kinase; MAPK: Mitogen activated protein kinase.

tion of ROS, which contributes to oxidative stress and a wide range of diseases. Furthermore, ROS can act as an intracellular second messenger or chemoattractant to enhance the level of cytokines, resulting in the aggravation of pancreatitis[38]. Studies indicate that pro-inflammation cytokines such as IL-1β, IL-6 and TNF-α mediate the local or systemic manifestations of acute pancreatitis. IL-1β and TNF-α released from activated pancreatic macrophages respond to local tissue damage. Locally, these cytokines may aggravate the severity of acute pancreatitis. Systemically, IL-6 can increase the capillary permeability and accelerate the leukocyte adherence, leading to multiple organ failure (Figure 2)[27].

NF-κB and Janus kinase/signal transducers and activators of transcription

NF-κB, a member of the Rel family of transcription factors, can regulate the activation of cellular stress-related genes or early response genes such as growth factors, cytokines, adhesion molecules, and acute-phase proteins[39,40]. The Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathway was relevant to the immune response mediated by numerous cytokines and non-immune response mediated by hormones and growth factors. The JAK/STAT pathway activated by the family of cytokine receptors regulate a variety of biological processes, such as immune response, cell survival, differentiation, proliferation and oncogenesis[41]. Recently, reports indicated that cerulein could activate the JAK2/STAT3 pathway through NOX in pancreatic acinar cells[27].

NOX may be the source of ROS in pancreatic acinar cells during pancreatitis. ROS can induce expression of cytokines, apoptosis, NF-κB and JAK/STAT pathway activation, thus regulating the inflammation and apoptosis in pancreatic acinar cells. Consequently, NOX, NF-κB and JAK2/STAT3 may be involved in the pathogenesis of acute pancreatitis[27]. Inflammation and apoptosis in pancreatic acinar cells during pancreatitis may be alleviated by inhibition of NOX, NF-κB and JAK/STAT through suppression of inflammatory cytokines, apoptosis and caspase-3 activity. Ju *et al*[23] found that NOX inhibition suppresses STAT3-DNA binding, JAK2/STAT3 activation and TGF-β1 level in AR42J cells stimulated by cerulein. Therefore, ROS may activate NF-κB to induce cytokine production in pancreatic acinar cells through activation of NOX during pancreatitis[21]. NOX, NF-κB and JAK/STAT may be potential targets for treatment of acute pancreatitis.

Mitogen activated protein kinase and tyrosine protein kinase

Recently, studies found that mitogen activated protein kinase (MAPK) and tyrosine protein kinase (TPK) might be involved in NOX signal transduction pathway. ROS induced by the family of NOX can cause protein phosphorylation and cell apoptosis directly or indirectly.

In the direct way, ROS mediate the activation of the MAPK pathway and TPK pathway to promote protein phosphorylation in pancreatic acinar cells. ROS activate the signal transduction pathway which consists of different MAPK family members probably owing to the activation of the upstream ERK1/2 kinase pathway. ROS stimulate TPK signaling pathway through increasing the TPK activity, thereby promoting protein tyrosine phosphorylation and affecting signal transduction to regulate cell proliferation, differentiation, metabolism and apoptosis. Inhibition of NOX or ROS significantly reduced the p38MAPK signaling cascade[42]. Activation of the MAPK signaling pathway including SAPK/JNK, ERK1/2 and p38 by ROS induce cell apoptosis. The activation of the MAPK pathway is mainly dependent on the inhibition of tyrosine phosphatase by ROS[43].

In the indirect way, ROS reduce phosphatase activity, decrease protein dephosphorylation, and thus indirectly increase protein phosphorylation. ROS injure DNA, lipid and protein, thus indirectly inducing apoptosis. In some cases, NOX family can also inhibit cell apoptosis through ROS, which activate the pathway of NF-κB and Akt/ASK1, thereby reducing cell apoptosis[44].

NOX ACTIVATION IN DIFFERENT PANCREATIC CELLS INVOLVED IN THE PATHOGENESIS OF PANCREATITIS

Phagocytes

In support of the involvement of oxygen free radicals in acute pancreatitis, studies have addressed the possibility that the severity of pancreatitis can be reduced by inhibiting the activity of oxygen-derived free radicals[45]. ROS could have different origins, and the role of the NOX system in neutrophils but not pancreatic acinar tissue is originally considered essential. The phagocytic NOX is a multicomponent enzyme complex that is composed of membranous and cytosolic proteins in the resting cell. During activation, approximately 10% of cytosolic proteins including $p47^{phox}$ and $p67^{phox}$ are phosphorylated and translocate to the cell membrane to form active catalytic complexes with $p22^{phox}$ and $gp91^{phox}$, resulting in the generation of ROS[4]. Intrapancreatic trypsin activation and acinar cell trypsin-activation peptide (TAP) labeling induced by high dose cerulein were significantly decreased in neutrophil depleted rats. NOX deficient mice displayed attenuation of the cerulean-induced trypsin activation, while myeloperoxidase (MPO) deficient mice did not. Neutrophils have been considered to be implicated in pathologic activation of digestive enzymes by infiltrating the pancreas in acute pancreatitis, which is mediated by products of NOX[28].

Evidence suggests that inflammatory cell infiltration is an early and vital event in acute pancreatitis, which will lead to local and systemic complications[46]. Many of the pathological failures of acute pancreatitis may be a consequence of the overstimulation of leukocytes[47]. The argument put forward was that once pancreatitis has been initiated, chemoattractants for polymorphonuclear leukocytes, macrophages and platelets are released, possibly *via* the action of oxygen derived free radicals. The chemoattractants induce leukocytes and macrophages to adhere to the endothelium of the postcapillary venule and to migrate into the interstitial spaces. Stimulus-secretion coupling causes synthesis of a range of enzymes including elastase, cathepsins, phospholipase A_2, phospholipase C, platelet-activating factor (PAF) and MPO. When the quantity of material to be digested is excessive, phagocytosis may become so vigorous that the contents of leukocyte and macrophage granules are spilled outside the cell where they increase the severity of inflammation. As a result, large amounts of oxygen-derived free radicals are produced and may exceed the capacity of superoxide dismutase (SOD) and catalase to inactivate them[48].

Pancreatic acinar cells

ROS and apoptosis can be observed in pancreatic acinar cells in cerulein induced pancreatitis[49,50]. NADPH has been considered to be the major source of ROS in pancreatitis[18,21,22]. Oxidative stress induced inflammation and apoptosis have been implicated in pancreatitis[51,52]. Cerulein induced the expression of apoptosis-inducing factor (AIF). AIF is located in the mitochondrial membrane of pancreatic acinar cells. During apoptosis, AIF translocates from mitochondria to the cytoplasm and then enters into the nucleus, resulting in nuclear DNA aggregation and breakage to induce apoptosis of pancreatic acinar cells[53,54]. Antisense oligonucleotides (AS ODN) transfection or Ca^{2+} chelator treatment decreased the expression of AIF induced by cerulein in AR42J cells. These results suggested that intracellular Ca^{2+} increase and NOX activation might be the upstream events of AIF expression, which result in cerulein induced apoptosis of AR42J cells[18,55].

The activation of NOX was inhibited and the production of ROS was decreased when cerulein-stimulated pancreatic acinar cells were treated with Ca^{2+} chelator, which indicates that Ca^{2+} activate NOX and ROS. Transfection with AS ODN for NOX subunits $p22^{phox}$ and $p47^{phox}$ can inhibit the ROS generation, illustrating that NOX mediates the production of ROS. The apoptotic indices including apoptotic genes bax and p53, DNA fragmentation, caspase 3 activity, TUNEL staining and cell viability were inhibited by treatment with Ca^{2+} chelator or AS ODN transfection, indicating that NOX regulates ROS-induced apoptosis in a Ca^{2+} dependent manner in pancreatic acinar cells[22]. Diphenyleneiodonium (DPI), an inhibitor of NOX, reduces the AIF expression and caspase-3 activation, and thus inhibits apoptosis of AR42J cells[16]. During the stimulation with cerulein, the increase of NOX accelerates the formation of ROS in cells and mitochondria, thus further inducing the apoptosis of acinar cells[56,57]. ROS generated by pancreatic acinar cells stimulated with bile acids or cerulein can

induce apoptosis and, at the same time, induce pancreatitis[58-60].

Research indicates that JAK2/STAT3 activation and increases of MAPKs and TGF-β1 induced by administration of cerulein were inhibited by AS ODN transfection in AR42J cells, which shows that NOX can activate JAK2/STAT3, MAPKs and TGF-β1[23]. NOX may regulate the production of cytokines by activating NF-κB in AR42J cells stimulated with cerulein. Rebamipide, an antiulcer agent, can scavenge ROS and decrease the level of superoxide[61,62]. Transfection with AS ODN for NOX subunits or administration of DPI or rebamipide inhibited cerulein induced NF-κB activation and IL-6 expression[21]. Cerulein also could produce large amounts of ROS to activate NF-κB and thus stimulate the expression of cytokines in freshly isolated pancreatic acinar cells without inflammation[63].

Numerous studies have shown that increases of ROS and peroxidation products are accompanied with endogenous antioxidant depletion in the early stage of pancreatitis. Many preclinical antioxidant treatments, including genetic manipulation, significantly reduce pancreatic injury and inflammation[1,64-66]. However, randomized clinical trials of antioxidants have produced conflicting results[67], and treatment of pancreatitis with antioxidants has even been discontinued because of adverse events[68]. Moreover, several studies indicated that NOX was only present in neutrophils but not in pancreatic acinar cells[28,69].

PSCs

PSCs are the major fibrogenic cells in chronic injury of the pancreas, which encircle the acinus[70,71]. PSCs account for approximately 4% of the total pancreatic cells[72]. PSCs are quiescent in normal pancreas and can be identified by the character of vitamin A containing lipid droplets in the cytoplasm. When chronic pancreatitis happens, PSCs are activated and transformed into myofibroblast-like cells. As a result, intracellular lipid droplets disappear and α-smooth muscle actin (α-SMA) and extracellular components such as fibronectin and collagen arise[19,73]. Besides, PSCs may be involved in the pathogenesis of acute pancreatitis[72]. Therefore, suppression of PSC activation is a potential target to treat pancreatic inflammation and fibrosis.

Studies showed that $p22^{phox}$, $p47^{phox}$, NOX1, $gp91^{phox}$ (NOX2), and NOX4 were expressed in rat quiescent and culture-activated PSCs as well as human activated PSCs, while $p67^{phox}$ and NOX3 were not detected. NOX activator 1 was present in human PSCs, while NOX organizer 1 was not detected. NOX can activate PSCs, which can be verified by DPI inhibition experiments. Studies showed that DPI could inhibit the activation of PSCs, that is, to inhibit proliferation, chemokine production, α-SMA and collagen expression. Platelet-derived growth factor BB (PDGF-BB) promoted proliferation of rat PSCs, which was inhibited by DPI in a dose-dependent manner, showing that NOX underlies the PDGF induced PSC proliferation. DPI decreased the chemokine production, which indicates that NOX also regulates the production of chemokines. DPI decreased the levels of α-SMA and collagen, once again, proving that NOX activate PSCs. DPI also inhibited interleukin 1β (IL-1β) and PDGF induced activation of MAPKs in PSCs, and this evidence indicates that NOX mediates the activation of MAPKs induced by IL-1β and PDGF in PSCs[19].

FUTURE RESEARCH ON THE PATHOGENESIS OF PANCREATITIS IN NOX

Accumulated evidence suggested that ROS induced by NOX play a significant role in pancreatitis. The activation of ROS mediates the activation of many cytokines[56,57]. ROS can induce cell apoptosis through direct and indirect pathways[43,44]. ROS induced by bile acids and cerulein can promote apoptosis of pancreatic acinar cells[18,69]. NOX is usually induced by cerulein, inflammatory factors, cytokines and growth factors as well as other stimuli in pancreatic acinar cells and PSCs. NOX can generate ROS, which in turn increase cytokines levels downstream to initiate the next activation cycle. The positive feedback of activation process might be one of the causes of pancreatitis. Although many scholars have made a great deal of research about the pathogenic mechanisms of NOX in the inflammation of pancreatic acinar cells and stellate cells, the relative importance of different pathogenic mechanisms of NOX in the pathogenesis of pancreatitis, the relationship between various pathogenic mechanisms of NOX, the specific pathways involved in each mechanism of NOX in pancreatitis, and the feasibility of NOX targeted therapy applied to pancreatitis are all needed to be studied in the future.

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TOPIC HIGHLIGHT

WJGP 5th Anniversary Special Issues (4): Barrett's

Barrett's oesophagus: Evidence from the current meta-analyses

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Abstract

Guidelines have been published regarding the management of Barrett's oesophagus (columnar-lined oesophagus). These have examined the role of surveillance in an effort to detect dysplasia and early cancer. The guidelines have provided criteria for enrolment into surveillance and some risk stratification with regard to surveillance interval. The research basis for the decisions reached with regard to cancer risk is weak and this manuscript has examined the available data published from meta-analyses up to 25th April 2013 (much of which has been published since the guidelines and their most recent updates have been written). There were 9 meta-analyses comparing patients with Barrett's oesophagus to control populations. These have demonstrated that Barrett's oesophagus is more common in males than females, in subjects who have ever smoked, in subjects with obesity, in subjects with prolonged symptoms of gastro-oesophageal reflux disease, in subjects who do not have infection with Helicobacter pylori and in subjects with hiatus hernia. These findings should inform public health measures in reducing the risk of Barrett's oesophagus and subsequent surveillance burden and cancer risk. There were 8 meta-analyses comparing different groups of patients with Barrett's oesophagus with regard to cancer risk. These have demonstrated that there was no statistically significant benefit of antireflux surgery over medical therapy, that endoscopic ablative therapy was effective in reducing cancer risk that there was similar cancer risk in patients with Barrett's oesophagus independent of geographic origin, that the adenocarcinoma incidence in males is twice the rate in females, that the cancer risk in long segment disease showed a trend to be higher than in short segment disease, that there was a trend for higher cancer risk in low-grade dysplasia over non-dysplastic Barrett's oesophagus, that there is a lower risk in patients with *Helicobacter pylori* infection and that there is a significant protective effect of aspirin and statins. There were no meta-analyses examining the role of intestinal metaplasia. These results demonstrate that guidance regarding surveillance based on the presence of intestinal metaplasia, segment length and the presence of low-grade dysplasia has a weak basis, and further consideration should be given to gender and helicobacter status, ablation of the metaplastic segment as well as the chemoprotective role of aspirin and statins.

Key words: Barrett esophagus; Esophageal neoplasms; Meta-analysis; Review; Systematic

Core tip: The presence of intestinal metaplasia on biopsy has been regarded as a necessity for enrolment

in a surveillance programme for Barrett's oesophagus and surveillance intervals have been based on segment length and the presence or absence of dysplasia. Evidence from meta-analyses supports male gender and negative *Helicobacter pylori* infection status as important markers of cancer risk and of the role of aspirin, statins and ablation of the Barrett's segment to reduce cancer risk. The evidence from meta-analyses supporting segment length and dysplasia as markers of cancer risk is poor and for intestinal metaplasia has not been shown.

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INTRODUCTION

Barrett's columnar-lined oesophagus is a metaplastic change to the squamous mucosa of the oesophagus associated with gastro-oesophageal reflux disease[1]. Guidelines concerning management of patients with Barrett's oesophagus have been published with recommendations on the control of pathological reflux and on periodic surveillance of this pre-malignant condition[2-4]. There has been a rapid increase in the number of meta-analyses published, with over half published in the last 5 years and an increase in the focus of these on pharmacotherapy and reflux control to reduce cancer incidence, associations with smoking and obesity as well as new estimates on cancer incidence. In an attempt to examine the available best evidence since these guidelines were published/updated (in 2013[2], 2011[4] and 2008[3]), this review has conducted a systematic review of the currently published meta-analyses to aid clinicians and patients in optimum decision making for the risk assessment and management of Barrett's oesophagus.

RESEARCH

A search was made of the Pubmed database for the search terms "Barrett's oesophagus" and "meta-analysis". The full search terms are listed in Table 1 with publication dates up to and including 25th April 2013 (including epublication). Papers were included in the analysis if the type of study was a meta-analysis of previously published data concerning Barrett's oesophagus in human subjects and published in English language. Studies were included if they compared subjects with Barrett's oesophagus to control groups or compared different groups of patients with Barrett's oesophagus with respect to cancer risk. Studies were then categorized into the following groups: (1) comparison of patients with Barrett's oesophagus to control groups; and (2) comparison of different groups of patients with Barrett's oesophagus with regard to cancer risk. Where the papers retrieved did not contain meta-analyses, but useful observations were presented, these have been described in this manuscript, but not included in the results tables.

The literature search yielded 50 papers. Of these papers, 10 were excluded after retrieving the abstracts and 6 after retrieving the full papers (2 were letters concerning meta analyses, 1 examined cell culture lines rather than studying human subjects, 4 were in foreign language-3 German and 1 Spanish, 1 was a systematic review without a meta-analysis, 1 was an economic review without a meta-analysis, 2 were reviews only, 3 did not contain a meta-analysis comparing any different groups and 2 were single studies). There were 34 remaining studies and the full manuscript of each was obtained. Eleven studies were excluded as they examined oesophageal cancer compared to control groups without an examination of a comparative risk in Barrett's oesophagus. Three examined diagnostic techniques only and have been excluded. Two examined the risk of adenocarcinoma development within high-grade dysplasia and were excluded. One examined the association of Barrett's oesophagus with colonic tumours (which demonstrated the increased risk of colonic tumours and colorectal cancer in subjects with Barrett's oesophagus[5]).

There were no studies comparing cancer risk in patients with Barrett's oesophagus to control groups. The remaining 17 studies are examined below.

The retrieved studies spanned the last 10 years. As would be anticipated with the growing popularity of meta-analyses, over half of the eligible studies were published since the beginning of 2010. The United States and United Kingdom guidelines were most recently updated in 2013[2], 2011[4] and 2008[3]. With the time required for preparation of these guidelines, this indicates that only a handful of the meta-analyses had been published sufficiently early for their results to be incorporated in the compilation of the American College of Gastroenterology guidelines and a limited number into the American Gastroenterological Association guidelines. In general, the guidelines have not examined differences in cancer risk between individuals beyond segment length, presence of intestinal metaplasia and dysplasia.

Comparison of patients with Barrett's oesophagus to control groups

There were 11 papers comparing patients with Barrett's oesophagus to control groups, usually taken from the general population, but also other endoscopic populations including those with reflux disease but no Barrett's oesophagus. These studies examined gender, smoking habits, obesity, symptom association, presence of *Helicobacter pylori* (*H. pylori*), presence of hiatus hernia and pattern of proton pump inhibitor usage. Of these, 9 were meta-analyses (Table 2).

Gender: The association between male gender and Barrett's oesophagus was demonstrated by Cook *et al*[6]. They

Table 1 Search terms

{"barrett's oesophagus" (All Fields) OR "barrett esophagus" (MeSH Terms) OR ["barrett" (All Fields) AND "esophagus" (All Fields)] OR "barrett esophagus"(All Fields) OR ["barrett's" (All Fields) AND "esophagus" (All Fields)] OR "barrett's esophagus" (All Fields)} OR {"barrett's oesophagus" (All Fields) OR "barrett esophagus" (MeSH Terms) OR ["barrett"(All Fields) AND "esophagus" (All Fields)] OR "barrett esophagus"(All Fields) OR ["barrett's" (All Fields) AND "esophagus" (All Fields)] OR "barrett's esophagus"(All Fields)}	AND	["meta-analysis" (Publication type) OR "meta-analysis as topic" (MeSH Terms) OR "meta-analysis" (All Fields)]

Search strategy: "Barrett's esophagus" or "Barrett's oesophagus".

Table 2 Meta-analyses comparing patients with Barrett's oesophagus to control groups

Subject	Ref.	Comparison	Group	Studies	Results	Outcome
Gender	Cook *et al*[6], 2005	Gender	Barrett's	32	M:F Ratio 1.96:1 (95%CI: 1.77, 2.77)	Higher M:F ratio in Barrett's oesophagus and reflux oesophagitis than in non-erosive reflux disease
			Erosive reflux disease	28	1.57 (95%CI: 1.40, 1.76)	
			Non-erosive reflux disease	14	0.72 (95%CI: 0.62, 0.84)	
Smoking	Andrici *et al*[7], 2013	Ever smoking	Barrett's *vs* GORD	20	OR, 1.18 (95%CI: 0.75, 1.86)	Cigarette smoking associated with increased risk of Barrett's oesophagus
			Barrett's *vs* non-GORD	27	OR, 1.44 (95%CI: 1.20, 1.74)	
Obesity	Cook *et al*[8], 2008	BMI	Barrett's *vs* GORD	9	OR, 0.99/kg per m^2 (95%CI: 0.97, 1.01)	Barrett's oesophagus associated with higher BMI than control but not GORD
			Barrett's *vs* general population	3	OR, 1.02/kg per m^2 (95%CI: 1.01, 1.04)	
	Kamat *et al*[9], 2009	Obesity (BMI ⩾ 30 *vs* BMI < 30)	Barrett's *vs* control (BMI ⩾ 30 *vs* BMI < 30)	9	OR, 1.35 (95%CI: 1.15, 1.59)	Barrett's oesophagus associated with being overweight and obese
		Overweight (BMI ⩾ 25 *vs* BMI < 25)	Barrett's *vs* control	8	OR, 1.49 (95%CI: 1.24, 1.80)	
	Kubo *et al*[10], 2013	Waist circumference	Highest *vs* lowest quartiles	4	Males OR, 2.24 (95%CI: 1.08, 4.65) Females OR, 3.75 (95%CI: 1.47, 9.56)	Barrett's oesophagus associated with higher waist circumference but not BMI
		BMI		4	No significant association	
Symptoms of gastro-oesophageal reflux	Taylor *et al*[11], 2010	Symptoms of GORD	All Barrett's *vs* controls	26	OR, 2.90 (95%CI: 1.86, 4.54)	Symptoms of GORD associated with all Barrett's oesophagus, more strongly with long segment Barrett's oesophagus than with short segment Barrett's oesophagus
			Short segment Barrett's *vs* controls	12	OR, 1.59 (95%CI: 1.07, 2.38)	
			Long segment Barrett's *vs* controls	11	OR, 4.16 (95%CI: 2.43, 7.12)	
Helicobacter pylori	Wang *et al*[12]	Helicobacter pylori infection rate	Barrett's oesophagus *vs* all controls	12	OR, 0.74 (95%CI: 0.40, 1.37)	Similar helicobacter pylori infection rate in Barrett's oesophagus to all controls but lower than in endoscopically normal controls
			Barrett's oesophagus *vs* endoscopically normal	9	OR, 0.50 (95%CI: 0.27, 0.93)	
	Fischbach *et al*[13], 2012	Helicobacter pylori infection rate	Barrett's oesophagus *vs* all controls	49	RR, 0.46 (9%CI: 0.35, 0.60)	Lower helicobacter infection rate in patients with Barrett's oesophagus compared to controls
		Cag A Helicobacter pylori infection rate	Barrett's oesophagus *vs* all controls	7	RR, 0.38 (95%CI: 0.19, 0.78)	
Hiatus hernia	Andrici *et al*[14], 2012	Hiatus hernia presence	Barrett's oesophagus *vs* all controls	31	OR, 3.94 (95%CI: 3.02, 5.13)	Hiatus hernia associated with Barrett's oesophagus and more strongly associated with long-segment Barrett's oesophagus

OR: Odds ratio; BMI: Body mass index; CI: Confidence interval.

examined data from studies on Barrett's oesophagus, erosive reflux disease and non-erosive reflux disease. The overall male: female ratio in Barrett's oesophagus was 1.96 and was similar in erosive reflux disease, but higher than

in non-erosive reflux disease.

Cigarette smoking: The association between cigarette smoking and diagnosis of Barrett's oesophagus was examined by Andrici *et al*[7]. They included a variety of different study designs and control subjects. They demonstrated that having ever smoked was associated with Barrett's oesophagus compared to control subjects who did not have gastro-oesophageal reflux disease or to population-based controls. There was no significant association when compared to controls with gastro-oesophageal reflux disease. There was a dose-related relationship with a higher number of pack-years smoked associated with increased risk of Barrett's oesophagus. The relationships were similar for current, former and ever smokers.

Obesity: Three studies examined the association between obesity and Barrett's oesophagus. Cook *et al*[8] examined studies which compared Barrett's oesophagus to those with reflux disease (those with unknown histology and those with histologically-proven oesophagitis) in 9 studies and to the general population in one study. Their results were similar for all comparison groups with no association noted with obesity and Barrett's oesophagus compared to gastro-oesophageal reflux disease, but in 3 studies comparing Barrett's oesophagus to control subjects there was a small statistically significant association between Barrett's oesophagus and higher body mass index. Kamat *et al*[9] showed that obesity was associated with Barrett's oesophagus and comparing patients who were either overweight or obese showed similar results. More recently, Kubo *et al*[10] showed that from 4 case-control studies that there was no clear association between BMI and Barrett's oesophagus, but that there was an increased risk of Barrett's oesophagus with higher waist circumference.

Symptoms: One study by Taylor *et al*[11] examined the association of Barrett's oesophagus with symptoms of gastro-oesophageal reflux. This analysis included 26 published studies (the majority of which were case-control) and demonstrated that symptoms of gastro-oesophageal reflux were associated with the diagnosis of Barrett's oesophagus, strongly with long segment Barrett's oesophagus and that there was a weaker association with short-segment Barrett's oesophagus.

***Helicobacter pylori*:** Wang *et al*[12] showed that there was no overall difference in *H. pylori* infection between patients with Barrett's oesophagus and control subjects (taken from blood donating populations and subjects with normal findings on endoscopy). When patients with Barrett's oesophagus were compared to those with normal endoscopy only, Barrett's oesophagus was associated with lower rate of *H. pylori* infection. With further data available, Fischbach *et al*[13] found that there was a strong negative association between the presence of *H. pylori* and Barrett's oesophagus. There were a smaller number of studies which examined the effect of virulent Cag A positive *H. pylori* with similar results.

Hiatus hernia: Andrici *et al*[14] examined the relationship between Barrett's oesophagus and hiatus hernia. Barrett's oesophagus was strongly associated with the presence of hiatus hernia compared to all controls, a significant association when compared to the control group of patients with gastro-oesophageal reflux disease and stronger association compared to control subjects without gastro-oesophageal reflux disease. The relationship was stronger for long segment Barrett's oesophagus than for short segment Barrett's oesophagus.

Pattern of proton pump inhibitor usage: There were 2 studies reported in the analysis of Hungin *et al*[15], but this was not undertaken as a meta-analysis. They analysed medication possession rates in patient with Barrett's oesophagus to those with gastro-oesophageal reflux disease and demonstrated higher adherence in those with Barrett's oesophagus. The self-reported adherence was also higher in patients with Barrett's oesophagus than subjects with gastro-oesophageal reflux disease in one of the included studies.

Comparison of different groups of patients with Barrett's oesophagus with regard to cancer risk

There were 12 studies which examined for differences in adenocarcinoma incidence in different groups of patients with Barrett's oesophagus. These studies looked at treatment for control of gastro-oesophageal reflux, endoscopic ablation of the metaplastic segment, demographic factors, segment length, dysplasia, enzyme polymorphisms, infection with *H. pylori* and drugs taken for other conditions. Eight of these studies contained meta-analyses (Table 3).

Treatment of gastro-oesophageal reflux and endoscopic ablation

Corey *et al*[16] examined the question of whether a surgical antireflux procedure was of benefit in reducing cancer risk in patients with Barrett's oesophagus. The cancer incidence was not significantly different between medical and surgical therapy and when the earlier medical cohorts were excluded (those prior to the proton-pump era), the cancer incidence in the medical group remained similar (0.43% per annum) to patients treated with antireflux surgery.

Li *et al*[17] examined randomized controlled trials of medical, surgical and endoscopic therapy for Barrett's oesophagus. There was one study of medical *vs* surgical therapy[18] which showed no significant difference in cancer incidence between patients treated by medical and surgical therapy (5% and 3% respectively), however there was a significantly lower risk of dysplasia development in the surgical arm (2%) compared to the medical arm (20%). There were three studies included of endo-

Table 3 Meta-analyses comparing cancer risk in different groups of patients with Barrett's oesophagus

Subject	Ref.	Comparison	Group	Studies	Results	Outcome
Medical *vs* surgical treatment of reflux	Corey *et al*[16]	Antireflux surgery *vs* medical treatment	Antireflux surgery	34	18 cancers/4678 patient-years (0.38% per annum)	No significant difference in cancer risk between medical and surgical antireflux therapy
			Medical therapy		26 cancers/4906 patient-years (0.53% per annum)	
Endoscopic ablative therapy *vs* surveillance	Wani *et al*[25]	Non-dysplastic Barrett's oesophagus	Surveillance	45	5.98/1000 patient-years	Endoscopic ablative therapy is effective in reducing adenocarcinoma risk in patients with non-dysplastic Barrett's oesophagus, low-grade dysplasia and high-grade dysplasia compared to surveillance alone
			Endoscopic ablative therapy	49	1.63/1000 patient-years	
		Low-grade dysplasia	Surveillance	16	16.98/1000 patient-years	
			Endoscopic ablative therapy	21	1.58/1000 patient-years	
		High-grade dysplasia	Surveillance	4	65.8/1000 patient-years	
			Endoscopic ablative therapy	28	16.76/1000 patient-years	
Demographic factors	Thomas *et al*[26]	Location	United Kingdom	13	7/1000 patient-years	Cancer incidence similar in all geographic areas
			United States	16	7/1000 patient years	
			Europe	10	8/1000 patient-years	
			Australia and New-Zealand	2	5/1000 patient-years	
	Yousef *et al*[27]	Gender	Males	6	10.2/1000 patient-years	Cancer incidence in males is double the rate in females
			Females	5	4.5/1000 patient-years	
Segment length	Thomas *et al*[26]	Segment length	Short segment	6	2.8/1000 patient-years	Trend for lower risk in short segment Barrett's oesophagus (P = 0.25)
			Long segment	6	7.8/1000 patient-years	
	Yousef *et al*[27]	Segment length	Short segment	6	6.1/1000 patient-years	Similar risk in short and long segment disease
			Long segment	26	6.7/1000 patient-years	
Dysplasia	Thomas *et al*[26]	Low-grade dysplasia as a confounding factor	Presence of low-grade dysplasia at index endoscopy	15	P = 0.23	No significant confounding effect on cancer incidence in meta-regression analysis
Helicobacter pylori	Rokkas *et al*[30]	All *Helicobacter pylori*	Cases	10	253/757 (34.3%)	*Helicobacter pylori* associated with lower rate of oesophageal cancer OR, 0.52; (95%CI: 0.37, 0.73)
			Controls	10	1398/2788 (50.1%)	
		Cag A *Helicobacter pylori*	Cases	6	120/462 (26%)	Cag A *Helicobacter pylori* associated with lower rate of oesophageal cancer OR, 0.51; (95%CI: 0.31, 0.82)
			Controls	6	774/1936 (40%)	
Non-steroidal Anti-inflammatory drugs	Wang *et al*[31]	Aspirin and NSAIDs *vs* controls		3	RR 0.64 (95%CI: 0.42, 0.96)	Lower risk of adenocarcinoma in patients taking aspirin or NSAIDs
Statins	Alexandre *et al*[33]	Statins *vs* controls		2	RR, 0.53 (95%CI: 0.36, 0.78)	Protective effect of statins *vs* controls
	Singh *et al*[36]			5	RR, 0.57; (95%CI: 0.44, 0.75)	
Statins and NSAIDs	Singh *et al*[36]	Combined statins and NSAIDs *vs* neither		2	0.28; (95%CI: 0.14, 0.56)	Protective effect of NSAIDs and statins higher than either individually

NSAIDs: Nonsteroidal antiinflammatory drugs; OR: Odds ratio; CI: Confidence interval.

scopic ablative therapy *vs* medical therapy for patients with dysplasia. The studies were heterogenous in their designs and outcome measures. Photodynamic therapy was superior to PPI in reducing the area of Barrett's epithelium[19] and eradication of dysplasia in patients with low-grade dysplasia[20] and high-grade dysplasia[20]. Overholt *et al*[20,21] also showed a lower rate of progression of high-grade dysplasia to cancer in the PDT group. There was one study[22] comparing endoscopic ablation of the metaplastic mucosa (with argon plasma coagulation) after antireflux surgery and showed a trend for superior endoscopic regression of the Barrett's segment after the

ablation, but no difference in cancer incidence[20]. In ablation of the metaplastic mucosa, 3 studies demonstrated that overall argon plasma coagulation was superior to photodynamic therapy with ablation rates of 59.0% and 27.5% respectively [odds ratio (OR), 3.46, 95% confidence interval (CI): 1.67, 7.81]. These studies did not examine long-term cancer incidences. There were 2 studies comparing argon plasma coagulation to multipolar electrocoagulation which demonstrated similar rates of successful ablation of the metaplastic segment (78.6% in patients treated with multipolar electrocoagulation and 64.4% treated with argon plasma coagulation) and again no long-term data on cancer incidence.

Fayter *et al*[23]examined the evidence from 11 randomised controlled trials of photodynamic therapy for Barrett's oesophagus. The trials were heterogeneous in their design, the protocol of therapy used, the patients studied (most studies examined patients with high-grade dysplasia, but some had low-grade dysplasia, non-dysplastic epithelium or a combination of histological findings) and outcome measures. The conclusions drawn from this systematic review were: (1) it was not possible to determine whether there was a significant clinical difference between photodynamic therapy and argon plasma coagulation and which would be the most appropriate treatment; (2) photodynamic therapy was more effective than omeprazole alone in producing long-term ablation of high-grade dysplasia and slowing/preventing progression to cancer; (3) Photodynamic therapy with 5-ALA as the photosensitising agent was more effective than placebo in producing regression of dysplasia and reduction in the area of Barrett's epithelium in patients with low-grade dysplasia; (4) photodynamic therapy with 5-ALA may be more effective than with Photofrin; (5) optimal treatment for patients without dysplasia had yet to be determined; and (6) side effects were similar between 5-ALA and Photofrin with higher levels of photosensitivity with Photofrin.

Rees *et al*[24]examined randomized controlled studies only. They demonstrated that in the 3 studies which examined H_2 receptor antagonists to proton pump inhibitors: cancer risk, eradication of dysplasia or complete regression of the metaplastic segment were not reported. There was a trend towards a reduction in the areas of metaplastic mucosa (but not the length of the Barretts' segment) with PPI. There were no new studies available on antireflux surgery *vs* medical therapy, argon plasma ablation, argon plasma coagulation *vs* multipolar electrocoagulation or argon plasma coagulation *vs* photodynamic therapy since Li *et al*[17].

Wani *et al*[25] compared the rate of development of adenocarcinoma in published series of patients with non-dysplastic Barrett's oesophagus, low-grade dysplasia and high-grade dysplasia comparing cohorts treated with endoscopic ablative therapy to those in surveillance programmes without ablation of the mucosa. They found that there were significantly lower rates of adenocarcinoma incidence in the cohorts treated with ablative therapies compared to the control cohorts. The differences were significant for examinations of non-dysplastic Barrett's oesophagus, low-grade dysplasia and high-grade dysplasia.

Demographic factors: Thomas *et al*[26] showed that age did not influence cancer risk from 41 studies of 9469 patients undergoing surveillance (36635 patient-years follow-up). There was also no significant difference in cancer incidence depending on geographic origin of the included studies. Yousef *et al*[27] showed that the incidence of adenocarcinoma in males was double the rate in females.

Segment length: Thomas *et al*[26] showed that from 6 studies including 960 patients with long-segment Barrett's oesophagus (4130 patient-years of follow-up) and 258 patients (1074 patient-years of follow up) that 32 of the 35 cancers which developed were in long segment Barrett's oesophagus (but this did not reach statistical significance). Yousef *et al*[27] reported a cancer incidence of 0.67% per annum in long segment Barrett's oesophagus and a similar incidence (0.61%) in short segment Barrett's oesophagus in 30 studies.

Dysplasia: Thomas *et al*[26] did not demonstrate an increased cancer risk associated with dysplasia over non-dysplastic Barrett's oesophagus. Desai *et al*[28] examined specifically patients without dysplasia at baseline, but there was no comparison cohort in this study and it has subsequently been excluded from this review.

Intestinal metaplasia: The question of the importance of intestinal metaplasia for cancer risk was not specifically examined by any of the meta-analyses.

Enzyme polymorphisms: Bull *et al*[29] examined enzyme polymorphisms in case-control studies and found an association between Barrett's oesophagus and GSTP1 homozygotes for the Ile105 variant (OR, 1.50, 95%CI: 1.16, 1.95). This genetic variant results in increased IgE and immune-mediated inflammation. There was no other significant association with Barrett's oesophagus and a variety of metabolic gene polymorphisms[29].

***Helicobacter pylori*:** Rokkas *et al*[30] showed similar results in studies of oesophageal cancer to those of Barrett's oesophagus with a negative association between the presence of *H. pylori* and oesophageal cancer. The results were similar in studies of Cag A *H. pylori*.

Other medications

The reduction in cancer risk with aspirin and non-steroidal anti-inflammatory drugs was examined in 3 cohort studies by Wang *et al*[31]. They demonstrated that there was a trend towards lower cancer risk in patients taking aspirin and non-steroidal anti-inflammatory drugs, however 2 case-control studies were excluded for unclear

reasons.

Rees *et al*[24] reported one study[32] comparing celecoxib to placebo and found no difference in cancer risk at 2 years (3/49 and 3/51 patients respectively).

The effects of statins on the risk of oesophageal adenocarcinoma in Barrett's oesophagus were examined by Alexandre *et al*[33], who found two prospective cohort studies. The first was a multicentre study from the Netherlands of 570 patients and demonstrated a hazard ratio of 0.46 (95%CI: 0.21, 0.99) and in patients taking statins and non-steroidal anti-inflammatory drugs the hazards ratio was 0.22 (95%CI: 0.06, 0.85)[34]. Nguyen *et al*[35] examined 812 patients in a case-control cohort in the Veterans Affairs Healthcare System and showed an incidence density ratio of 0.56 (95%CI: 0.36, 0.86) for patients with Barrett's oesophagus taking statins.

Singh *et al*[36] also demonstrated a protective effect of statins in their meta-analysis of 5 studies and a greater protective effect of combining statins with non-steroidal anti-inflammatory drugs with respect to oesophageal cancer risk.

Decision to enrol in surveillance

The American College of Gastroenterology and American Gastroenterological Association have defined Barrett's oesophagus as any length of recognisable columnar mucosa which demonstrates intestinal metaplasia at biopsy[3,4], maintaining the dogma that intestinal metaplasia is necessary for malignant risk on the basis that in many cohort studies intestinal metaplasia has been demonstrated adjacent to adenocarcinoma of the oesophagus. The ACG acknowledge the difficulties associated with sampling error in the detection of intestinal metaplasia and also exclude "ultra-short" segments (< 1 cm) due to poor interobserver reliability of recognition. The BSG broadly agrees with this definition[2] and whilst there is no requirement for the presence of intestinal metaplasia for diagnosis, on the basis of the higher cancer risk in subjects with intestinal metaplasia in the Northern Ireland pathology database cohort[37] and low rate of development of high-grade dysplasia and adenocarcinoma in the Danish pathology database cohort which only included subjects with intestinal metaplasia[38], surveillance is only recommended if intestinal metaplasia is detected during the either the index or the first surveillance endoscopy in patients with short segment (< 3 cm) metaplasia. The rationale for this is that it is felt that the risks of endoscopy probably outweigh the benefits. Both guidelines have excluded very short segments or tongues of metaplasia due to difficulties in clinical assessment rather than on the basis of a proven low risk of complications and there are no good data to support or refute these assertions. The evidence from meta-analyses concerning the role of segment length and intestinal metaplasia is discussed below.

The ACG recommend that the consideration for beginning a surveillance programme should include age, likelihood of survival over the next 5 years, patient's understanding of the process and its limitations for the detection of cancer and the willingness of the patient to adhere to the recommendations.

The ACG supports surveillance of Barrett's oesophagus as in 7 retrospective series the survival in cancers was improved over those detected outside of surveillance programmes. There has not yet been a trial published demonstrating benefits of surveillance in a prospective fashion, however the BOSS study (endoscopic surveillance *vs* endoscopy at time of need) remains underway at present[39].

The ACG, AGA and BSG recommend 4-quadrant biopsies taken every 2 cm throughout the metaplastic segment at index endoscopy and surveillance (if no dysplasia has been previously detected or other macroscopic lesions are present). This biopsy protocol has not yet been tested in a meta-analysis. The difficulties involved in adequately sampling the tissue at risk and variability in histopathological interpretation of the tissue examined should be subject to further studies beyond the initial work done by Levine *et al*[40].

Risk stratification and frequency of surveillance

The ACG recommend that the first two endoscopies are undertaken within a year and if no dysplasia is detected then the surveillance interval is 3 years. If low-grade dysplasia is detected then surveillance interval should be within 6 mo. This recommendation was based upon a poor level of evidence from cohort studies and expert opinion[3].

The BSG note that risk factors for cancer development include the presence of intestinal metaplasia (3 × compared to no intestinal metaplasia), low-grade dysplasia (5.67 × non-dysplastic Barrett's oesophagus), male gender (2 × that of females), smoking (2 × non-smokers). They note that longer segment lengths were associated with a trend to increased risk and no relationship was demonstrated with alcohol consumption and obesity[2].

The ACG and AGA stratify risk based only on the presence of dysplasia after the diagnosis of Barrett's oesophagus and that further work to assess the extent of dysplasia and develop biomarkers is required[3,4]. The BSG note that in future, surveillance intervals will take into account all of the socio-demographic risk factors and characteristics of the Barrett's segment as well as biomarker panels[2] . Until such algorithms are developed, surveillance frequency is based on dysplasia and length only. The ACG also note that a randomised controlled trial to assess the impact of surveillance is required. The BSG also incorporate segment length and allow for consideration of other risk factors (see above)[2]. The BSG have lengthened the recommended surveillance interval for non-dysplastic Barrett's oesophagus (based upon the recent lower cancer incidence estimates) in line with the AGA and allowed some further individualised risk stratification to be incorporated into the frequency of surveillance and in line with the ACG, the interval for low-

grade dysplasia is 6 mo. The AGA recommend surveillance of low-grade dysplasia in 6-12 mo. Inflammatory atypia is difficult to distinguish from true dysplasia[2,41] and the guidelines recommend repeat biopsy after treatment with acid suppression[3] and expert pathological review of biopsies which are dysplastic or have changes indefinite for dysplasia[2-4].

The evidence from the meta-analyses in supporting intestinal metaplasia and low-grade dysplasia as markers of increased risk of malignancy is poor with no significant difference demonstrated in patients with low-grade dysplasia at index endoscopy[26] and no papers on the necessity for the detection of intestinal metaplasia to confer a malignant risk.

Evidence for difference in risk dependent on segment length is also poor with only trends demonstrated[26,27] and it is only on weak evidence that decisions on consideration of surveillance as well as surveillance interval are made on these features.

There is greater evidence for a lower risk of oesophageal cancer development in patients who have *H, pylori* infection[30] and for a higher risk in males over females[27].

What steps to minimise risk of developing Barrett's

The ACG notes that older Caucasian males with chronic reflux symptoms are the group with the highest prevalence of Barrett's oesophagus and there were no direct recommendations from the ACG to reduce the risk of development of Barrett's oesophagus[3]. The BSG state that the known risk factors are male gender, older age and history of reflux symptoms as well as an association with white race, higher waist: hip ration and abdominal circumference. There is a less clear relationship with obesity as measured by body mass index and cigarette smoking. The BSG also note the small degree of familial clustering[2]. The AGA go one step further in recommending consideration of screening for Barrett's oesophagus in patients with multiple risk factors for oesophageal adenocarcinoma (age 50 years or older, male sex, white race, chronic gastro-oesophageal reflux disease, hiatal hernia, elevated body mass index and intra-abdominal distribution of body fat).

The published meta-analyses have demonstrated that the significant risk factors associated with Barrett's oesophagus are male gender[6], smoking[7], obesity[8-10], prolonged symptoms of gastro-oesophageal reflux[11], absence of *H. pylori* infection[12,13] and the presence of hiatus hernia[14]. Age has not been demonstrated to influence cancer risk in the meta-analyses[26].

Minimisation of risk of cancer development in Barrett's

The ACG, AGA and BSG did not recommend fundoplication over medical therapy to reduce cancer development[2-4] and this review supports this strategy[16,17], however there were encouraging data concerning reduction in risk of development of dysplasia with surgical therapy over acid suppression therapy[18].

The question of ablation of the metaplastic mucosa is a complex one requiring further examination, however there are promising results[25] and the SURF trial comparing radiofrequency ablation to surveillance in low-grade dysplasia remains underway[42].

The ACG note that a meta-analysis did demonstrate a lower risk of cancer development in patients taking non-steroidal anti-inflammatory drugs[43] and that the ASPECT study (a randomised study of aspirin and low and high-dose esomeprazole) remains underway[44]. The ACG, AGA and BSG did not recommend chemoprevention with aspirin or non-steroidal anti-inflammatory drugs. The ACG cites two cohort studies demonstrating a lower risk of dysplasia development in patients taking PPI therapy, but no evidence to support a reduction in cancer development[3]. The BSG recommendations are similar to those of the ACG and also do not advocate acid suppression drugs as chemopreventive agents[2], but they are effective in symptom control.

The AGA note that the patients may derive benefit from aspirin if they have cardiovascular risk factors for which aspirin therapy is indicated, but that neither the use of aspirin or non-steroidal anti-inflammatory drugs are recommended solely to prevent oesophageal adenocarcinoma and that the evidence to support the use of PPI therapy to reduce the risk of cancer and dysplasia is indirect and not been proven in a long-term controlled trial[4].The results from the meta-analyses of Alexandre *et al*[33] and Singh *et al*[36] showing the protective effect of aspirin and in particular the effect in conjunction with statins is exciting and may form the basis for effective chemoprevention in the future.

CONCLUSION

The evidence to support the current decisions to enrol patients with Barrett's oesophagus in surveillance programmes and surveillance interval are based on weak evidence on the clinical outcome of features of the metaplastic segment. Further consideration should be given to the role of gender and helicobacter status in examining cancer risk as well as the role of aspirin and statins in chemopreventive strategies and ablation of the metaplastic segment. Public health programmes should also examine measures to reduce the associations of Barrett's oesophagus, notably, smoking and obesity. The relevance of male gender and absence of helicobacter infection should also be considered.

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TOPIC HIGHLIGHT

WJGP 5th Anniversary Special Issues (5): Cholangiocarcinoma

Review to better understand the macroscopic subtypes and histogenesis of intrahepatic cholangiocarcinoma

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Abstract

Intrahepatic cholangiocarcinoma is macroscopically classified into three subtypes, mass-forming-type, periductal infiltrating-type, and intraductal growth-type. Each subtype should be preoperatively differentiated to perform the valid surgical resection. Recent researches have revealed the clinical, radiologic, pathobiological characteristics of each subtype. We reviewed recently published studies covering various aspects of intrahepatic cholangiocarcinoma (ICC), focusing especially on the macroscopic subtypes and stem cell features to better understand the pathophysiology of ICC and to establish the valid therapeutic strategy.

Key words: Intrahepatic cholangiocarcinoma; Combined hepatocellular-cholangiocarcinoma; Hepatic progenitor cells; Macroscopic subtype

Core tip: We reviewed recently published studies covering various aspects of intrahepatic cholangiocarcinoma (ICC), focusing especially on the macroscopic subtypes and stem cell features to better understand the pathophysiology of ICC and to establish the valid therapeutic strategy.

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INTRODUCTION

The Liver Cancer Study Group of Japan has applied the same TNM staging system used for hepatocellular carcinoma (HCC) to that for intrahepatic cholangiocarcinoma (ICC)[1]. A recent increase in the number of surgically resected cases of ICC has clarified some characteristics inherent in this disease. The most prominent feature of ICC is that of the macroscopic findings reflecting its growth patterns. ICC is grossly classifiable into mass-forming (MF), periductal infiltrating (PI), and intraductal growth (IG) types[2]. The MF type presents as a gray to gray-white, firm and solid mass in the hepatic parenchyma, and of these three subtypes, MF-type ICC is the most common (59%). The PI type shows spreading of the carcinoma along the portal tracts with stricture of the central bile ducts and dilation of the peripheral bile ducts. The IG type presents as a papillary tumor within the dilated bile duct lumen. Some IG-type ICCs are considered to be an intraductal papillary neoplasm of the bile duct. This classification system provides useful information during surgery (Figure 1). For example, the efficacy of hilar resection is not emphasized except in the case of PI-type ICCs. This macroscopic classification cannot be applied to HCC. Therefore, studies focusing on the association of the macroscopic subtypes with biological behavior, clinical features, and radiologic

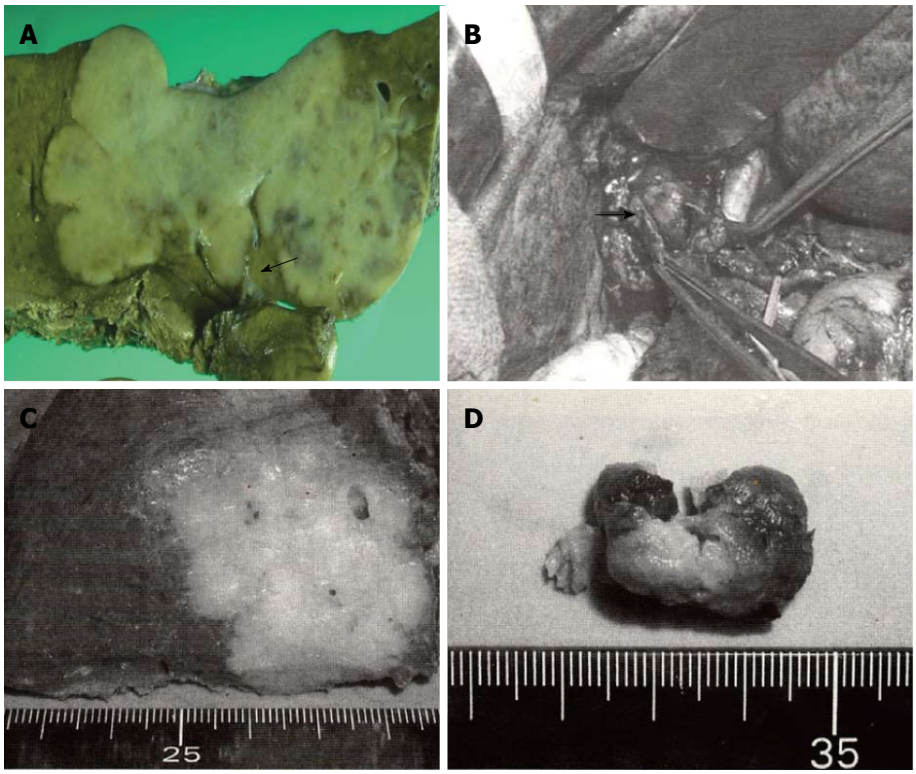

Figure 1 Some intraductal growth-type intrahepatic cholangiocarcinomas are considered to be an intraductal papillary neoplasm of the bile duct, this classification system provides useful information during surgery. A: Gross feature of mass-forming (MF) + periductal infiltrating (PI)-type intrahepatic cholangiocarcinoma (ICC) obtained by left hepatectomy with bile duct resection. The carcinoma spreads along the hilar biliary tree (arrow) in communication with a white firm mass; B-D: Operative findings and resected specimens of MF + intraductal growth (IG)-type ICC. The common hepatic duct is incised, and the soft tumor comprising the intraductal components is easily removed (arrow) without infiltration to the ductal wall; C: Hepatic anterior segmentectomy is performed for MF components; D: IG components are composed of tan-colored soft tissues with necrosis.

findings are needed to establish the therapeutic strategy for ICC. Although the macroscopic features are prominent in ICCs, another aspect of ICCs, in which ICCs cannot be discussed independently of other primary liver cancers, exists. Recent histopathologic and immunohistochemical studies have reported that hepatic progenitor cells (HPC) or stem cells play important roles in liver carcinogenesis including both HCCs and ICCs, supporting the hypothesis that HCCs and ICCs share a common evolutionary origin[3,4]. In 2010, the World Health Organization (WHO) established a new classification system of combined hepatocellular-cholangiocarcinoma (cHCC-CC) based on the presence of stem-cell features[5]. According to this new system, cHCC-CCs are classified into two major subtypes, classic type and subtypes with stem-cell features. Subtypes with stem-cell features are further subclassified into three types: typical type, intermediate-cell type, and cholangiocellular type. In addition, recent reports showed that some cases of HCCs and ICCs are associated with hepatic stem cells. However, little is known about the clinical significance of stem cells in ICCs. This review summarizes recently published studies (from 2011 to 2013) covering various aspects of ICC and cHCC-CC, focusing especially on the macroscopic subtypes and stem-cell features.

CLINICAL STUDIES OF ICC

Recent clinical researches of ICC are summarized in Tables 1 and 2[6-24]. The association between macroscopic subtypes and survival rate and lymph node metastasis has been discussed ever since the macroscopic subtype was established. IG-type ICCs have a favorable outcome because this tumor type shows intraductal growth without invasiveness[2]. Of the three subtypes, MF+PI-type ICCs have the highest incidence of lymph node metastasis (50% to 73%)[15] and are associated with the lowest 5-year survival rate (0% to 19.4%). PI-type and MF-type have relatively favorable outcomes when lymph node metastasis or hilar invasion is absent.

Over the most recent 3 years, 19 studies have been published (Tables 1 and 2). Most of these studies describe the poor prognostic factors of resected cases of ICC. The most significant prognostic factor is lymph node metastasis. However, whether routine lymph node dissection improves postoperative survival is still unclear.

The literature on the macroscopic subtypes is very scant. Uchiyama *et al*[15] and Uno *et al*[17] reported that the PI type showed significantly better survival than the MF and MF+PI types, supporting the results of previous reports. The difference in malignant potential between

Table 1 Clinical study of intrahepatic cholangiocarcinoma

Ref.	*n*	Survival rate (%)	MST (mo)	Prognostic factor
Marubashi *et al*[6]	111	59.7 (3 yr)	-	IM, Hilar inv, LN
Guglielmi *et al*[7]	145	-	19 (LN+), 42 (LN-)	LNR > 0.25, LN
Zhu *et al*[8]	37	-	-	CA19-9, Low prealbmin
Dhanasekaran *et al*[9]	105	-	16	V
Wang *et al*[10]	367	-	-	CEA, CA19-9, Size, V
De Rose *et al*[11]	79 (MF)	-	-	Doubling time < 70 d
Sulpice *et al*[12]	87	-	-	BT, Maj, Size, V, IM
Ribero *et al*[13]	434	39.8 (5 yr)	-	LN, CA19-9, IM
Liu *et al*[14]	132	-	-	Por, CA19-9, Dis(-)
Uchiyama *et al*[15]	334	-	-	Shown in Table 2
Chen *et al*[16]	64	32 (3 yr)	-	LN, PN, Size
Uno *et al*[17]	273	-	-	Shown in Table 2
Morine *et al*[18]	22	-	-	Shown in Table 2
Jiang *et al*[19]	102	-	-	CA19-9, IM
Murakami *et al*[20]	44	47 (5 yr)	-	LN
Clark *et al*[21]	4893	8.4 (5 yr, LN+) 25 (5 yr, LN-)	-	LN
de Jong *et al*[22]	449	31 (5 yr)	27	IM, V, LN
Li *et al*[23]	115	-	-	Cirrhosis
Chen *et al*[24]	320	-	-	-

MST: Median survival time; Prognostic factor: Factor for poor prognosis; IM: Multiple tumors or intrahepatic metastasis; Hilar inv: Hilar invasion; LN: Lymph node metastasis; LNR: Rate of the positive lymph node metastasis; CA19-9: Elevated serum carbohydrate antigen 19-9; CEA: Elevated serum carcinoembryonic antigen; Size: Larger tumor size; V: Vascular invasion; BT: Blood transfusion during operation; Dis: Lymph node dissection; PN: Perineural invasion.

Table 2 Clinical studies of intrahepatic cholangiocarcinoma focused on the macroscopic subtypes

Ref.	*n*	Findings or conclusion
Uchiyama *et al*[15]	334	Lymph node metastasis: MF: 16%; IG: 0%; PI and MF + PI: 60% Survival rate (5 yr): MF: 26%; IG: 79.3%; PI and MF + PI: 19.4%
Uno *et al*[17]	273	Rate of PI-type: 7.9% The PI-type shows significantly better survival than MF- and MF + PI-type
Morine *et al*[18]	22	The PI-type shows a lower incidence of intrahepatic metastasis Routine lymph node dissection do not improve survival in MF-type

MF: Mass-forming type; IG: Intraductal growth type; PI: Periductal infiltrative type.

each subtype emphasizes the importance of the preoperative identification of each subtype.

RADIOLOGIC STUDIES OF ICC

Table 3 summarizes recent radiologic studies of ICC[25-30]. The typical enhancement pattern of ICC on CT and MRI is that of ringed enhancement in the early phase with central delayed enhancement, reflecting the abundant fibrous stroma in ICC. However, Kim *et al*[26] reported that 6 (30%) of 20 ICCs appeared as hypervascular lesions with washout in the delayed phase, resembling HCCs. In addition, Ariizumi *et al*[29] pointed out that MF-type ICCs with hypervascular-type pattern had more favorable prognosis than those with the typical enhancement pattern. The histopathological characteristics of hypervascular-type ICCs have not been clarified. Cholangiocellular carcinoma (CoCC), a subtype of ICC, has been reported to originate from the ductules, or canals of Hering, and appears as a hypervascular mass similar to HCC[31]. These results of recent radiologic studies suggest the possibility that some ICCs share the same origin with that of CoCC, *i.e.*, HPCs. Especially in MF-type ICCs, comparative studies between the enhancement patterns and histopathologic findings are needed for further exploration. However, these descriptions can be applied to only MF-type ICCs. Xu *et al*[28] reported the difference of enhancement patterns on contrast-enhanced ultrasonography between each subtype and demonstrated that most IG-type ICCs appeared as a mass showing homogenous hyperenhancement. This finding provides useful knowledge for preoperative differentiation between IG-type and PI-type ICC.

PATHOBIOLOGICAL STUDIES OF ICCs

During the most recent 3 years, many molecules have been identified as biomarkers for poor prognosis of ICCs (Tables 4-6)[31-71]. Among these, researchers have paid close attention to molecules associated with epithelial-mesenchymal transition (EMT)[32,38,53,55]. The

Table 3 Radiologic studies of intrahepatic cholangiocarcinoma

Ref.	*n*	Method	Findings or conclusion
Nanashima *et al*[25]	42	CT	Factor for poor prognosis: case showing arterial enhancement with lower attenuation
Kim *et al*[26]	20	MRI	6 (30%) of the 20 cases appeared as hypervascular lesions with washout on delayed phase
Kang *et al*[27]	50	MRI	Percentage of relative enhancement on hepatobiliary phase was significantly higher in moderately differentiated tumors than in poorly differentiated tumors and in patients without than in those with lymph node metastasis
Xu *et al*[28]	40	Contrast enhanced ultrasono-graphy	MF-type (n = 32): (1) peripheral rim-like hyperenhancement (n = 19); (2) heterogenous enhancement (n = 10); and (3) homogenous hyperenhancement (n = 3)
Ariizumi *et al*[29]	26	FDG PET	PI-type (n = 4): heterogenous enhancement (n = 4) IG-type (n = 4): (1) homogenous hyperenhancement (n = 3); and (2) heterogenous enhancement (n = 1) FDG PET was able to predict patient outcome after radioembolization treatment

CT: Computed tomography; MRI: Magnetic resonance imaging; FDG PET: ^{18}F-fluorodeoxy glucose positron emission tomography.

Table 4 Pathobiological studies of intrahepatic cholangiocarcinoma

Ref.	*n*	Method	Target	Conclusion
Gu *et al*[32]	85	IHC	E-cadherin	(-)por
			Beta-catenin	(-)por
			Vimentin	(-)por
Yan *et al*[33]	49	IHC	Smad4	(-)por, advanced stage, LN
Kamphues *et al*[34]	65	DNA-Cyto	DNA-index	(+)poor prognosis
Mano *et al*[35]	132	IHC	Roundabout-1	(-)Size, Ki67index, poor prognosis
			Slit-1	(-)PN, LN
Yin *et al*[36]	411	Serum	γ-glutamyl transferase	(+)V, LN, poor prognosis, incomplete encapsulation
Sulpice *et al*[37]	40	mRNA (Stroma)	Osteopontin	(+)poor prognosis
			TGFβ2	(+)poor prognosis
			Laminin	(+)poor prognosis
Zhou *et al*[38]	Cell line	mRNA Western	Notch-1	(+)EMT
Li *et al*[39]	173	IHC	CKAP4	(+)favorable prognosis
Nanashima *et al*[40]	38	IHC	CD44	(+)PI-type, poor prognosis
			Gli1	(+)poor prognosis
Nutthasirikul *et al*[41]	-	mRNA	Δ133p53/TA P53	(+)poor prognosis
	-	IHC	Mutantp53	(+)poor prognosis
Zhang *et al*[42]	33	mRNA Western	Capn4	(+)LN, advanced stage, Poor prognosis
Ding *et al*[43]	20	IHC	Integrinα6	(+)IM, Size, V, poor prognosis
		Cell	Integrinα6	(-)decrease of metastasis
Aishima *et al*[44]	134	IHC	Cox-2	(+)poor prognosis, LN
			iNOS	(-) LN
Chen *et al*[45]	61	IHC	IMP3	(+)Por, advanced stage, V poor prognosis, CA19-9

IHC: Immunohistochemistry; mRNA: Real-time polymerase chain reaction; Western: Western blotting; DNA-cyto: DNA image cytometry; Cell: Functional analyses using cell lines; CKAP4: Cytoskeleton-associated protein4; iNOS: Inducible nitric oxide synthase; IMP3: Insulin-like growth factor II mRNA binding protein.

close association between EMT and the progression of ICC was confirmed not only by immunohistochemistry but also by functional and comprehensive analyses. The fact that EMT induces progression of ICC led us to hypothesize that abundant fibrous stroma in ICCs play an important role in the invasive growth and metastasis of this cancer. In addition, Oishi *et al*[53] reported that activation of miR-200c induced a reduction in EMT and in the expression of neural adhesion molecule (NCAM). Given that NCAM is known to be a hepatic progenitor cell marker, a hypothesis that the hepatic progenitor cell markers and molecules associated with EMT are regulated by common upstream molecules can be proposed. Further functional analyses are needed to confirm this hypothesis.

The literature on the association between macro-

Table 5 Pathobiological studies of intrahepatic cholangiocarcinoma 2

Ref.	*n*	Method	Target	Conclusion
Shi *et al*[46]	138	IHC	DKK-1	(+)poor prognosis elevated sMMP9 and VEGF-C
		Cell	DKK-1	(-)decrease in cell migration and invasiveness (+)LN, Por, advanced stage, V
Yao *et al*[47]	96	IHC	Vimentin and N-cadherin	poor prognosis (+)MF-type
Zhou *et al*[48]	54	IHC	HBx-protein	well differentiated tumor (+)well differentiated tumor, IG-type
Choi *et al*[49]	46	IHC	CK20 MUC6	(+)favorable prognosis (+)Size, LN, V, advanced stage
Jeong *et al*[50]	43	IHC Cell	FABP-5 FABP-5	(-)decrease in cell proliferation and invasion (+)elevated serum CEA and CA
Tsai *et al*[51]	112	IHC	S100P	19-9 value, MUC2 positive poor prognosis (+)perineural invasion
	86	Sequencing	K-ras mutation miR-200c	poor prognosis (+)reduction of EMT
Oishi *et al*[53]	-	Microarray		reduction of NCAM1 expression
Liao *et al*[54]	-	Cell	HCV core protein Angiotensin	(+)enhanced NFAT expression (+)enhanced Angiotensin II receptor expression and fibrogenesis of cancerous stroma, metastasis
Okamoto *et al*[55]	-	Cell	II and SDF1	

DKK1: Dickkopf-related protein1; MMP: Matrix metalloproteinase; FABP-5: Fatty acid-binding protein 5; SDF1: Stromal cell derived factor 1; NCAM1: Neural cell adhesion molecule1; EMT: Epithelial mesenchymal transition; NFAT1: Nuclear factor of activated T-cells.

Table 6 Pathobiological studies of intrahepatic cholangiocarcinoma 3

Source	*n*	Method	Target	Conclusion
Li *et al*[56]	-	Tissues	miR-214	(-)increased expression of Twist(EMT -associated gene)
Gu *et al*[57]	123	IHC	IL-17cells (intratumoral)	(+)poor prognosis
Higashi *et al*[58]	63	IHC	MUC16	(+)poor prognosis
Gu *et al*[59]	83	IHC	E-cadherin Beta-catenin EGFR	(-)poor prognosis (-)V (+)Por
Wang *et al*[60]	77	IHC	P-70S6K 4EBP1	(+)Por (+)poor prognosis
Hirashita *et al*[61]	35	IHC	MMP-7	(+)poor prognosis
Srimunta *et al*[62]	55	IHC	ABCC-1	(+)poor prognosis
Morine *et al*[63]	35	IHC	HDAC	(+)advanced stage, LN poor prognosis
Wakai *et al*[64]	34	IHC	RRM1	(+)gemcitabine resistance
Larbcharoensub *et al*[65]	60	IHC	ABCG2	(-)poor prognosis, LN, Por
Lee *et al*[66]	101	IHC	PTEN P-AKT1 P-MTOR	(+)favorable prognosis (+)favorable prognosis (+)favorable prognosis
Dong *et al*[67]	108	IHC	Beclin1	(-)LN, poor prognosis
Shinozaki *et al*[68]	83	IHC	Claudin-18	(+)LN, PI-type, perineural invasion
Wakai *et al*[69]	34	IHC	NQO1	(-)Por, poor prognosis
Aishima *et al*[70]	110	IHC	S100P S100P(nuc)	(+)PI-type (+)LN, V
Zhou *et al*[71]	89	IHC	MAGE3/4	(+)larger tumor size, poor prognosis

EGFR: Epidermal growth factor receptor; P-70S6K: P70 ribosomal protein S6 kinase; 4EBP1: 4E-binding protein-1; ABCC-1: Adenosine triphosphate binding cassette C1; HDAC: Histone deacetylase; RRM1: Ribonucleotide reductase-M1; ABCG2: Adenosine 5′ triphosphate-binding cassetteG2; PTEN: Phosphatase and tensin homolog on chromosome ten; PAKT: Phosphorylated Akt; PMTOR: Phosphorylated MTOR; NQO1: Quinine oxidoredactase; MAGE: Melanoma antigen.

scopic subtypes and the expression of genes are very scant[48,49,68,70], similar to that in the clinical study literature. Shinozaki *et al*[68] reported that claudin-18 (CLDN18), a tight junction protein specific to the stomach and lung,

Table 7 Clinical studies of combined hepatocellular-cholangiocarcinoma

Source	*n*	Conclusion or findings
Yap *et al*[74]	11	Survival rate: 69.3% (3 yr)
Lee *et al*[75]	65	(1) The clinical characteristics of cHCC-CC are similar to those of HCC (2) Overall survival of cHCC-CC is similar to that of ICC
Yin *et al*[76]	113	(1) Findings similar to HCC: infection with hepatitis virus; presence of cirrhosis; elevated AFP levels (2) Findings similar to ICC: serum CA19-9 elevation; incomplete capsules; lymph node involvement (3) Survival rate: 41.4%(3 yr); 36.4% (5 yr) (4) Factors for poor prognosis: radical liver resection
Ariizumi *et al*[77]	44	(1) Survival rate: 24% (2) Median survival time: 15.4 mo
Yu *et al*[78]	14	(1) Clinical characteristics: hepatitis B virus infection: 13/14; elevated AFP levels: 11/14 (2) Median survival time: 7.9 mo (3) Stem cell markers (IHC): c-Kit 71.4%; CD90: 85.7%; CD133: 92.9%; CK19: 78.6%
Park *et al*[79]	21	Factor for poor prognosis: serum AFP levels
Park *et al*[80]	43	(1) median survival time: 34 mo (2) Survival rate: 18.1% (5 yr) (3) Factors for poor prognosis: Portal vein thrombosis; distant metastasis
Zhan *et al*[81]	27	(1) CK-7: 86.4%; CK19: 90.9% (2) Survival rate: 49.4% (3) Factors for higher recurrence: lymph node metastasis

AFP: Alpha-fetoprotein.

Table 8 Radiologic studies of combined hepatocellular-cholangiocarcinoma

Ref.	*n*	Methods	Conclusion or findings
Ijichi *et al*[82]	3	FDG -PET	(1) SUVmax value of three cHCC-CC cases: 9.9, 12.0, and 13 (2) Median SUVmax value of poorly differentiated HCC: 5.7
de Campos *et al*[83]	11	MRI	(1) 6/11 showed early ring enhancement with progressive enhancement in central portion. (2) 5/11 showed a diffuse heterogenous early enhancement. Characteristics findings of cHCC-CC: irregular shape and strong rim enhancement during early phase; absence of target appearance on hepatobiliary-phase
Hwang *et al*[84]	20	MRI	

cHCC-CC: Combined hepatocellular-cholangiocarcinoma; MRI: Magnetic resonance imaging; FDG-PET: ^{18}F-fluorodeoxy glucose positron emission tomography.

is highly expressed in precancerous lesions of biliary intraepithelial neoplasms and PI components of ICCs. CLDN18 has been reported to be expressed in various gastrointestinal cancer tissues and to be associated with morphogenesis of the histologic subtype and the specific mucin phenotype[72]. In addition, we previously reported the association between the expression of CLDN18 and intestinal-type differentiation in intraductal papillary-mucinous neoplasm of the pancreas[73]. Thus, there is considerable interest in the crucial role of CLDN18 in the development of PI-type morphology in ICCs.

RECENT RESEARCH ON cHCC-CC

There is a large dissociation in the postoperative survival rates of cHCC-CC reported in the recent researches[74-90] (Tables 7-9), probably because the case numbers are limited. In addition, cHCC-CC is associated with many factors that contribute to poor prognosis including lymph node metastasis, higher levels of serum AFP, and portal vein thrombosis, reflecting intermediate features of cHCC-CC between HCC and ICC (Figure 2). The intermingling of findings of cHCC-CCs are also demonstrated by radiologic studies. Based on the new WHO classification system of cHCC-CC, some immunohistochemical research highlighting the expression of HPC markers has been published in the past 3 years in which YAP1 and EpiCAM, are reported to be markers of poor prognosis. These molecules are mainly distributed across the intermediate- and cholangiocellular-type components. Kim *et al*[85] reported that YAP1 is localized in the transitional zone between HCC and ICC components. In addition, Akiba *et al*[87] demonstrated that vimentin is strongly expressed in intermediate-type cHCC-CC. Similar to their role in ICCs, HPC markers may also play a crucial role in the progression of cHCC-CC through EMT. These components may harbor biological instability resembling undifferentiated carcinoma that leads to invasive behavior. However, CoCC, a subtype of ICC, has been known to be a tumor with characteristics resembling those of HCC and to have a relatively favorable prognosis (Figure 3). Given that CoCC is also derived from HPCs[31], a contradictory point exists with regard to the role of HPCs in the progression of ICCs

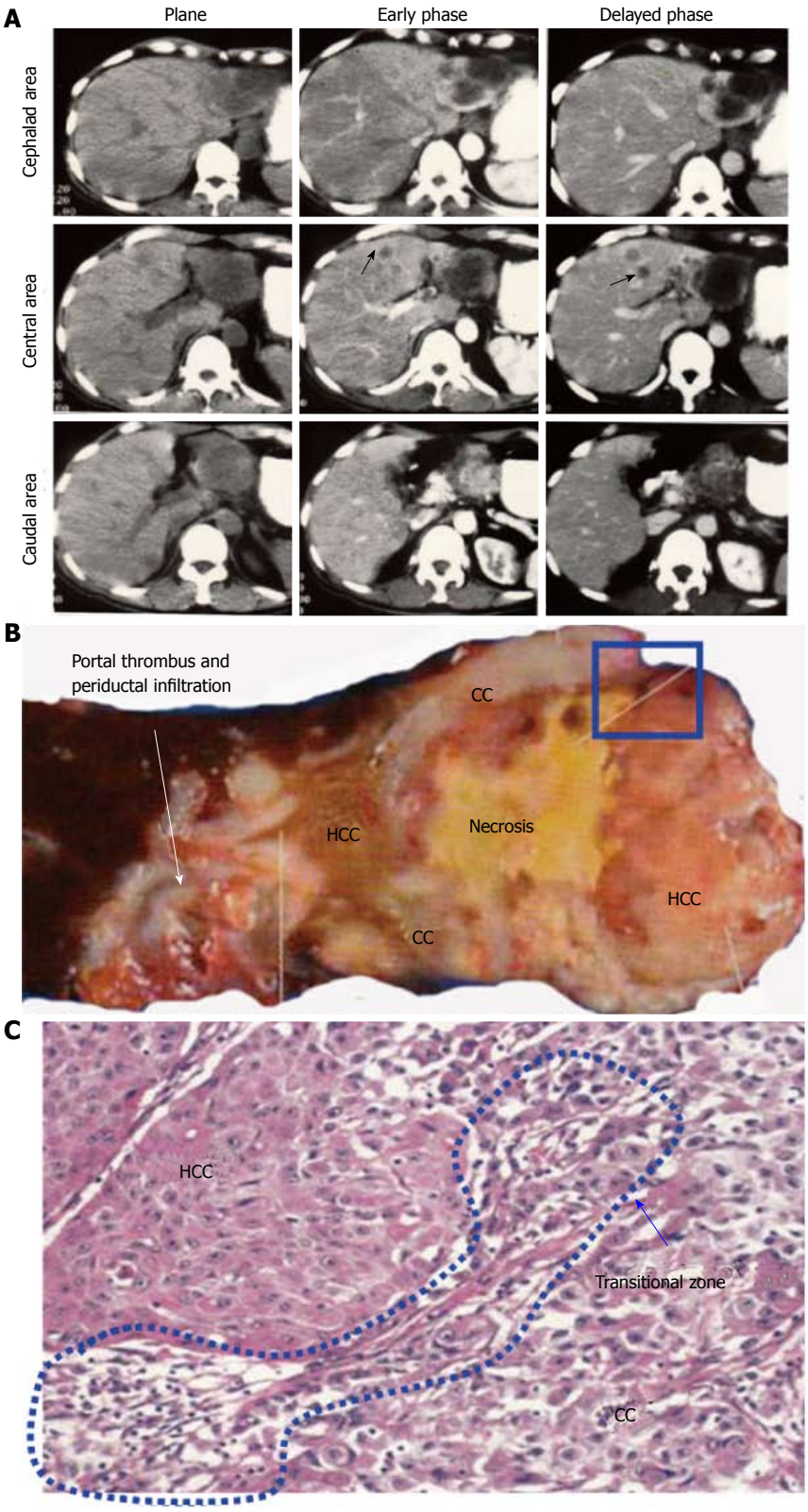

Figure 2 Case presentation of combined hepatocellular-cholangiocarcinoma. A: Preoperative computed tomography shows a large mass composed of two major components that replaces the lateral segment. The mass shows ringed enhancement in the delayed phase in the cephalad area and early enhancement with washout in the delayed phase in the caudal area. Intrahepatic metastases are observed in the S4 segment (arrow); B: Gross features of the resected specimen. Hepatocellular carcinoma (HCC) components are composed of tan-colored soft tissues. Cholangiocarcinoma (CC) components are composed of white firm tissues with central necrosis; C: Small round cells and fibrous stroma are observed at the boundary area between the HCC and CC components (blue flame in panel B).

and cHCC-CCs. We speculate that each HPC marker performs various functions involving progression and metastasis of ICCs and cHCC-CCs to a lesser or greater extent.

CONCLUSION

Recent research in ICC has revealed that each tumor shows different clinical and radiologic characteristics between the macroscopic subtypes. However, there are still many unclear points regarding the molecular mechanisms yielding these subtypes. It is of particular interest to identify the molecular markers inducing invasion, metastasis, and the macroscopic growth patterns of ICC. Many researchers have noted that HPC markers and EMT are involved in the progression of ICCs.

Table 9 Pathobiological studies of combined hepatocellular-cholangiocarcinoma

Ref.	*n*	Method	Target	Conclusion
Kim *et al*[85]	58	IHC	YAP1	(+): transition zone, poor prognosis
			EpiCAM	(-)favorable prognosis
			CK19	(-)favorable prognosis
Ikeda *et al*[86]	36	IHC	DLK1	(+)poor prognosis
Akiba *et al*[87]	54	IHC	CD56	(+): components apart from HCC
			c-Kit	(+): components apart from HCC
			EpiCAM	(+): components apart from HCC
			CD133	(+): intermediate type or cholangiolocellular type
			Vimentin	(+): intermediate type or cholangiolocellular type
Coulouarn *et al*[88]	152	Microarray	-	(1) TGFbeta and beta-catenin are identified as the two major signals in the progression of cHCC-CC/ (2) cHCC-CC shares the characteristics of poorly differentiated HCC. (+)poor prognosis
Cai *et al*[89]	80	IHC	PCNA	Both HCC and CC components of most Of the cHCC-CC express both AFP and CK19
Itoyama *et al*[90]	20	IHC	AFP and CK19	

cHCC-CC: Combined hepatocellular-cholangiocarcinoma; YAP1: Yes-associated protein 1; EpiCAM: Epithelial cell adhesion molecule; DLK1: Delta-like 1 homolog; PCNA: Proliferating cell nuclear antigen index in nontumor ductular reaction.

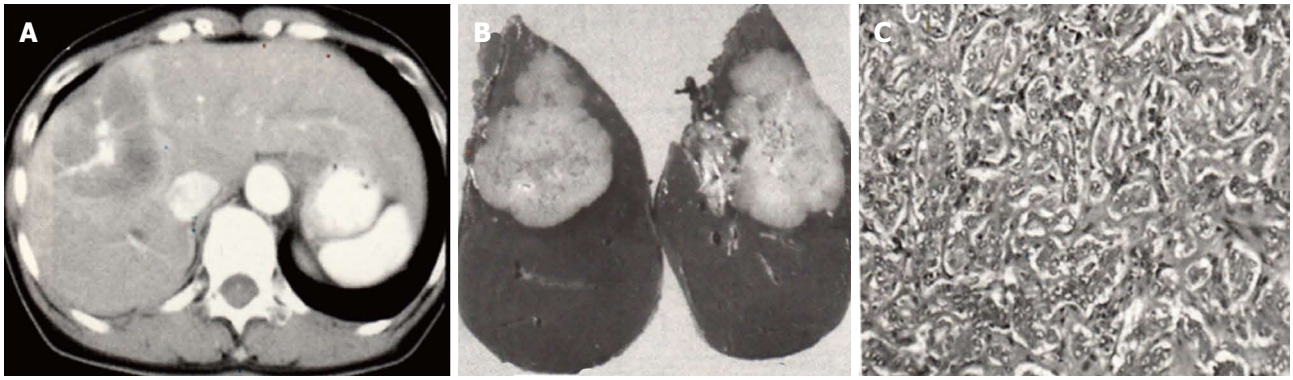

Figure 3 Resected case of cholangiocellular carcinoma. A: Computed tomography (CT) reveals a mass showing ringed enhancement with portal venous penetration; B: CT findings reflect the non-infiltrative growth of the tumor to the portal tract; C: Histopathologically, the size of the carcinoma cells is small, with the cells forming anastomosing patterns with abundant fibrous stroma.

Because most cHCC-CCs show MF-type morphology, we infer that HPC markers are closely associated with the morphogenesis and histogenesis of MF-type ICCs. Therefore, studies of ICC, and especially of its molecular pathology, should be designed in conjunction with those of cHCC-CC.

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TOPIC HIGHLIGHT

WJGP 5th Anniversary Special Issues (6): Crohn's disease

Laparoscopic surgery in the management of Crohn's disease

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Abstract

Crohn's disease is a chronic inflammatory bowel disease with surgery still frequently necessary in its treatment. Since the 1990's, laparoscopic surgery has become increasingly common for primary resections in patients with Crohn's disease and has now become the standard of care. Studies have shown no difference in recurrence rates when compared to open surgery and benefits include shorter hospital stay, lower rates of wound infection and decreased time to bowel function. This review highlights studies comparing the laparoscopic approach to the open approach in specific situations, including cases of complicated Crohn's disease.

Key words: Crohn's; Laparoscopy; Surgery; Colon; Ileum

Core tip: Laparoscopy is now increasingly used in cases of Crohn's disease. Recurrence rates are similar to that of open surgery and studies have shown benefits of decreased hospital stay as well as earlier bowel function. This review highlights several studies that looked at patients who underwent ileocolic and colon resections as well as more complicated cases of Crohn's.

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INTRODUCTION

Crohn's disease is an autoimmune disorder that causes chronic transmural inflammation of the gastrointestinal tract and makes up one of the two main components of inflammatory bowel disease[1]. The terminal ileum and proximal colon are the most frequently affected and initial diagnosis is made early, between the ages of 20-30[2]. Despite the advances in medical therapies with increasingly new immunomodulator use, the rate of refractory disease requiring surgery has not changed over the years[3]. Surgery is still common and up to 80% of patients with Crohn's disease will require an operation during their lifetime, with 15%-20% requiring an operation within the first year after diagnosis[4-6]. Of those patients that undergo surgery, studies have shown that approximately 40%-50% will likely need additional surgical intervention within 10-15 years[7,8]. The likelihood of a second surgery within one's lifetime is high, with several studies having identified the median age of first surgical resection to be in a patient's third decade[6,9].

Initially, laparoscopic surgery was not attempted for Crohn's disease due to the intraoperative characteristics that made a laparoscopic approach challenging. These findings often included extensive inflammation, enteric fistulae, thickened mesentery, and skip lesions throughout the bowel[10]. This belief has changed over time and laparoscopy has become increasingly accepted in patients with Crohn's disease as the use of laparoscopy in a majority of gastrointestinal procedures has become standard[11]. Crohn's patients are typically young and benefit from a laparoscopic procedure that reduces scar and adhesion formation. In addition, given their high risk of surgical recurrence, Crohn's patients benefit from

surgical approaches that maximize abdominal wall integrity[2,10,12,13].

This article review will evaluate surgical resections as well as common surgical scenarios commonly seen with Crohn's disease and compare the laparoscopic and open approaches. A search was conducted in the PubMed, Cochrane, MEDLINE, and Scopus libraries with the following individual and combined key words: Crohn's disease, laparoscopy, surgery, cost, colon, ileocolic, fistula, recurrent, small bowel, outcome, minimally invasive surgery, inflammatory bowel disease, randomized, metaanalysis. References cited in the articles retrieved were also searched in order to identify other potential sources of information. The results were limited to human studies available in English.

LAPAROSCOPIC ILEOCOLIC RESECTIONS

One of the first randomized trials comparing laparoscopic resections to open resections for refractory ileocolic disease was published in 2001 by Milsom *et al*[14] Sixty patients were randomized to undergo either laparoscopic or open procedures. The authors reported improved morbidity rates and hospital length of stay rates in the laparoscopic group, although the anastomotic leak rate was similar between the two groups. Length of surgery favored the open group. Long term follow up showed no difference between the groups in terms of disease recurrence rates.

A similar long term prospective study undertaken from 1999-2003 in the Netherlands showed similar results of no difference in overall disease recurrence between the laparoscopic and the open groups. Additionally, there were fewer incidences of small bowel obstruction and incisional hernias in the laparoscopic group. Overall, patient quality of life and cosmesis scores favored the laparoscopic group[15].

One of the weaknesses of these randomized prospective studies is that the overall number of patients treated was small. However, metaanalysis studies with a larger number of subjects show that these findings for laparoscopic surgeries are consistent. In a large metaanalysis by Tilney, data from over 15 different studies looking specifically at laparoscopic ileocolic resections was compiled. The analysis included 783 patients, 338 (43.2%) of which had undergone laparoscopic resection. The overall conversion rate to open surgery was 6.8%. As seen in earlier studies, overall surgery duration was longer in the laparoscopic group with a difference of 29.6 min. Perioperative complications and anastomotic leak rates were similar between the two groups. Benefits of laparoscopy were significantly shorter time till bowel function was regained and a shorter hospital stay by 2.7 d[16].

These findings are also supported by other smaller prospective and retrospective studies comparing open *vs* laparoscopic ileocolic resections in patients with Crohn's disease. There were no differences in morbidity and mortality. Furthermore lengths of time till return of bowel function and hospital stay were consistently shorter in the laparoscopic groups[17-20].

More recently, data reviewed from the National Surgical Quality Improvement Program from 2005-2009 compiled perioperative results from over 1900 ileocolic resections for Crohn's disease, 34% of which were performed laparoscopically. On multivariate analysis, the laparoscopic group was associated with an overall decrease in major and minor perioperative complications as well as a significant decrease in overall hospital stay by 1.08 d[21].

Long term studies following open and laparoscopic ileocolic resection patients showed no difference in recurrence rates[22,23]. In one study, the average time to recurrence was 60 mo in the laparoscopic group and 62 mo in the open group. Another study reported the average five year recurrence rates to be 29.1% in laparoscopic patients and 27.7% in open patients. Median times to recurrence were 48 and 56 mo, respectively. These times were not significant with a *P*-value of 0.9104. Of note, the laparoscopic group was found to have lower bowel obstruction rates over that time period[18].

LAPAROSCOPIC COLON RESECTIONS

Much of the literature focuses on laparoscopic surgery at the ileocolic region. Because Crohn's disease can affect any part of the gastrointestinal tract, other anatomical locations can pose different challenges. Acute colitis rates in Crohn's disease patients ranges from 5% to 10%[24]. Given the larger size of the colon and potentially broader thicker diseased mesentery of the colon, laparoscopic surgery for Crohn's colitis was slower to become accepted.

One of the earliest studies comparing minimally invasive surgery in Crohn's colitis to open surgery was in 2007. This study case matched 27 patients based on various patient factors including comorbidities and types of surgery, looking at patients only with disease in the colon. The authors found that although overall surgery was longer in the laparoscopic group, complications and estimated blood loss were the same in both groups. Length of hospital stay was significantly shorter in the laparoscopic group when 30 d readmissions were included[25].

Another study retrospectively looked at 92 patients with Crohn's disease that underwent minimally invasive colon resections. Forty-three cases (47%) were total colectomies, 17 (18%) were subtotal colectomies, 32 (35%) were segmental resections. There were 15 conversions to open resections, but conversions were not associated with longer hospital stay or increased postoperative complications. Five patients required reoperation, three for obstruction and two for anastomotic leak. The only prognostic factor for a complicated hospital course was evidence of perianal disease and 30-d mortality was zero[26].

One of the largest studies looking at laparoscopic colon resections in patients with Crohn's disease prospectively compared 55 laparoscopic resections to 70 open resections. The conversion rate to open resection of 10.9 was similar to that for ileocolic resections. Of note, 34.5% of patients who underwent laparoscopic surgery had had prior abdominal surgery as compared to 65.7% of the open group. This is one of the weaknesses of this study as surgeon preference dictated which procedure was performed. Although there was likely selection bias, the laparoscopic group was associated with similar benefits that were identified in the randomized studies for ileocolic resections. These benefits included less intraoperative blood loss, shorter hospital stays and quicker return of bowel function after surgery in the laparoscopic group[27].

LAPAROSCOPIC SURGERY FOR FISTULIZING DISEASE

Enteric fistulas are challenging complications in Crohn's patients as this finding often implies the presence of a large inflammatory mass, a history of prior surgeries, or use of steroids-all of which can make the surgery technically difficult. Surgery for enteric fistulas requires resection of the involved segment and primary anastomosis in the elective setting. Fistulas involving other organs are treated with bowel resection of the involved segment and primary repair of the other involved organ[10,28]. Some studies have cited intraoperative discovery of an intraabdominal abscess or fistula as an independent risk factor for conversion from a laparoscopic procedure[29]. In addition, a recent consensus conference was unable to recommend a laparoscopic approach for cases of complex Crohn's disease[30].

A laparoscopic approach in these patients with complicated Crohn's can be treacherous but as surgeons have become more skilled with laparoscopy, more studies have shown its feasibility. One retrospective review looked at 72 patients who underwent laparoscopic surgery for enteric fistulas. This study included enterocolic, ileo-ileal, enterocutaneous, ileovesical, colovesical, colocutaneous, and colovaginal fistulas. Prior abdominal surgery was present in 39.7% of the patients. Approximately 30% of the patients had multiple fistulas and 12.3% of those underwent multiple resections. The rate of conversion to open resection was low at 4.1% and overall morbidity was 11%[28].

In a more recent case-matched study 11 patients presenting with 13 fistulas were matched to 22 controls with non-fistulizing disease according to age, sex, nutritional state, steroid use, and type of laparoscopic resection[31]. Although the sample size was small, the authors were unable to show any difference in operative time, conversion rates, or morbidity rates between the two groups.

A larger prospective comparative study compared laparoscopic ileocolonic resections in patients with complex Crohn's disease (abscess and/or fistula) to patients without complex Crohn's disease. There was no significant difference in postoperative complications but overall operative time, conversion rates, and frequency of temporary stoma creation were all significantly increased in the complex Crohn's group[32]. These findings are suggestive of a more challenging operation, although the lack of increased morbidity demonstrates that a laparoscopic approach is still feasible. This is an area that needs to be continued to be studied.

LAPAROSCOPIC SURGERY FOR RECURRENT DISEASE

As indications for laparoscopic surgery in patients with Crohn's disease expands, its use in patients with recurrent disease seems natural. Several studies have compared laparoscopic resection to open resection for recurrent disease with no significant difference in surgical outcomes[33,34]. The reported conversion rates within these studies ranged from 6.7%-42% with the most common reasons for conversion being adhesions, intraoperative discovery of fistula/abscess, or need for associated bowel resection. In general, the conversion rate for recurrent Crohn's disease was similar to numbers seen in surgeries for initial disease. Only in one study was the conversion rate higher in the recurrent disease group and risk factors for conversion were age greater than 40, repeat resection for recurrent disease, and operative findings of an abscess[35].

Laparoscopic surgery is also possible in patients whose primary operation was a midline laparotomy. A recent study compared laparoscopic *vs* open surgery for patients with recurrent Crohn's disease where their primary surgery was a bowel resection through a midline laparotomy. The study was a retrospective case matched study comparing 26 patients who underwent laparoscopic resection to 26 patients that underwent open resection. Both groups had comparable demographics in terms of comorbidities and prior number of abdominal surgeries. Of note, the recovery benefits of shorter hospital stay and earlier return of bowel function that are seen in all other studies were not maintained in the laparoscopic group. However, there was a significant decrease in wound complication rates when compared to the open group[36].

ROLE OF SINGLE INCISION SURGERY IN CROHN'S DISEASE

As the role of laparoscopy has increased in patients with Crohn's disease, other advances such as single incision surgeries have also been studied in these patients. Of the few studies published, there is a significant amount of heterogeneity in terms of the technical aspects of the procedures and long term data is not available. Initial results though, show that the single incision approach is feasible without a large increase in complications and with the benefit of decreased postoperative analgesia.

Other studies have shown that complication profile is similar to laparoscopic surgery with the only advantage appearing to be the decreased number of trocar sites while all other factors were equivalent[8,37-39].

COST EFFECTIVENESS OF LAPAROSCOPIC SURGERY IN CROHN'S DISEASE

The overall cost of care for Crohn's disease continues to increase, with some estimates placing the annual cost in the United States anywhere from $10-15.9 billion and $2.1-16.7 billion in Europe[40-42]. These estimates are expected to increase as newer biologic drugs are increasingly available and used in management[43]. Of these costs, hospitalizations accounted for 53%-66% of the total in the United States with an average of $37459 per hospitalization[41].

Laparoscopy has the potential to decrease these costs per hospitalization as studies have shown that, when compared to open surgeries, laparoscopic surgeries reduce length of hospital stays and concomitant complications. A recent study comparing laparoscopic to open cases found the difference in hospital charges were significantly different, on average $27575 *vs* $38713 respectively[44]. These savings are consistent with those seen in colorectal cancer resections when comparing laparoscopic to open surgeries[45]. These savings can be potentially further reduced with the increasing adoption of single port surgery as well[46].

CONCLUSION

Current literature lacks a large number of randomized trials, but the consistent outcomes seen in the numerous retrospective studies and the small number of randomized studies shows that minimally invasive surgical approaches for Crohn's disease patients are both feasible and safe. It is important to remember that patient selection and surgeon experience are important factors for successful laparoscopic surgery. Complicated Crohn's cases with recurrent disease and enteric fistulas require knowledge of advanced laparoscopic techniques. The primary benefits of laparoscopic surgery over open surgery are quicker return to bowel function, decreased wound infection rates and shorter hospital stays. With no difference in recurrence rates seen, laparoscopy is emerging as the standard approach for patients with Crohn's disease for initial surgery, and even in select cases of patients with recurrent and complicated Crohn's disease.

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TOPIC HIGHLIGHT

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Pathophysiology of fistula formation in Crohn's disease

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Abstract

Fistulae represent an important complication in patient suffering from Crohn's disease (CD). Cumulative incidence of fistula formation in CD patients is 17%-50% and about one third of patients suffer from recurring fistulae formation. Medical treatment options often fail and also surgery frequently is not successful. Available data indicate that CD-associated fistulae originate from an epithelial defect that may be caused by ongoing inflammation. Having undergone epithelial to mesenchymal transition (EMT), intestinal epithelial cells (IEC) penetrate into deeper layers of the mucosa and the gut wall causing localized tissue damage formation of a tube like structure and finally a connection to other organs or the body surface. EMT of IEC may be initially aimed to improve wound repair mechanisms since "conventional" wound healing mechanisms, such as migration of fibroblasts, are impaired in CD patients. EMT also enhances activation of matrix remodelling enzymes such as matrix metalloproteinase (MMP)-3 and MMP-9 causing further tissue damage and inflammation. Finally, soluble mediators like TNF and interleukin-13 further induce their own expression in an autocrine manner and enhance expression of molecules associated with cell invasiveness aggravating the process. Additionally, pathogen-associated molecular patterns also seem to play a role for induction of EMT and fistula development. Though current knowledge suggests a number of therapeutic options, new and more effective therapeutic approaches are urgently needed for patients suffering from CD-associated fistulae. A better understanding of the pathophysiology of fistula formation, however, is a prerequisite for the development of more efficacious medical anti-fistula treatments.

Key words: Crohn's disease; Fistula; Tumor necrosis factor; Interleukin-13; Transforming growth factor; Epithelial to mesenchymal transition

Core tip: Fistulae represent an important complication in Crohn's disease (CD) patients. CD-associated fistulae originate from an epithelial defect due to destructive inflammation. Having undergone epithelial-to-mesenchymal transition (EMT), intestinal epithelial cells (IEC) penetrate into deeper layers of the gut wall causing further tissue damage finally forming connections to other organs or the body surface. EMT of IEC results in activation of matrix remodelling enzymes. Soluble mediators like TNF and IL-13 induce their own expression and expression of molecules associated with cell invasiveness. A better understanding of the pathophysiology of fistula formation is a prerequisite for developing more efficacious medical anti-fistula treatments.

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INTRODUCTION

Crohn's disease (CD) and ulcerative colitis are the two main forms of inflammatory bowel diseases (IBD) and are characterized by chronic intestinal inflammation. An important complication of CD is the formation of fistulae. This frequent clinical problem often causes a severely impaired quality of life in the affected patients. According to population-based studies, the cumulative incidence of fistula formation in CD patients is 17%-50%[1-3]. About 35% of patients suffer from at least one fistula during their disease course[2]. After 10 years of disease, the cumulative incidence for fistulas is reported to be up to 33% and after 20 years it is about 50%[2]. About one third of patients suffer from recurring fistulae[2]. In roughly 10% of the CD patients fistulae are the initial disease presentation and may precede the manifestation of intestinal disease by several years[1]. Therapeutic options are limited: Medical options include antibiotics, immunosuppressives (such as azathioprine or cyclosporine) and anti-TNF antibodies. Their clinical effect is often limited and despite medical treatment more than one third of patients suffers from recurring fistulae[4]. However, also surgical option do not always provide a definitive solution and permanent fistula closure can only be achieved in about 34% of CD patients[5].

MORPHOLOGICAL CHARACTERISATION OF CD FISTULAE

CD fistulae are found perianal in the majority of cases (54%), as well as entero-enteric (24%), recto-vaginal (9%) or in other locations, such as entero-cutaneous or entero-vesical[2]. On a histomorphologic level, CD-associated fistulae reveal a central fissure that penetrates through the lamina propria and muscularis mucosa into deeper layers of the underlying gut wall. In general, fistulae are lined by granulation tissue consisting of histiocytes and a dense network of tender capillaries. The lumen is frequently filled up by nuclear debris, erythrocytes as well as neutrophils and lymphocytes indicating non-specific acute or chronic inflammation. In more than 80% there are signs of moderate to severe acute inflammation. In CD fistulae, the wall of the fistulae is frequently infiltrated by $CD45RO^+$ positive T-cells, followed by a small band of $CD68^+$ positive macrophages and finally a dense infiltrate of $CD20^+$ B-cells. This is in contrast to non-CD fistulae, where often an intense infiltration by $CD68^+$ macrophages, but only a few $CD20^+$ B-lymphocytes and $CD45RO^+$ T-lymphocytes are observed[6].

Independent of the inflammatory infiltrate about one fourth of CD fistulae feature a lining epithelium central in the fistulising inflammation. Depending on the fistula location, this lining epithelium consists of flattened epithelial cells of the small intestine or colon without goblet cells or of a narrow squamous epithelium in cutaneous or perianal fistulae. The cells reveal tight junctions and a basement membrane. In "non-epithelialized" fistulae some areas are lined with myofibroblast-like cells, so-called transitional cells (TC). The region where the mucosal epithelial cells transform into the TC is called transitional zone. The TC are connected by gap junctions to each other and in certain areas a new basement membrane develops between TC and the underlying granulation tissue. The TC and the new basement membrane are connected by fibronexus. However, in adjacent areas there are also disordered myofibroblasts showing no gap junctions and a fragmented basement membrane[6].

ALTERED MIGRATORY POTENTIAL OF COLONIC LAMINA PROPRIA FIBROBLASTS

On a functional level, the migratory potential of colonic lamina propria fibroblasts (CLPF) in the intestine of CD patients is clearly less than in non-IBD or UC patients. Of note, mucosal fibroblasts derived from CD-associated fistulae reveal an even further reduced migratory potential what might contribute to decreased wound healing potential in this disease[7,8]. The decrease in the migratory potential of the CLPF can be induced by pro-inflammatory cytokines, such as TNF or IFN and is a persistent functional change since it is not reversible even after removing the cytokines. Additionally, it is accompanied by a decreased expression and phosphorylation, meaning activation, of the focal adhesion kinase (FAK)[7,9] which is a central regulator of cell migration[10]. Fibronectin is also an essential factor for the induction of migration of CLPF. In CD fistulae, the ED-A and ED-B isoforms of fibronectin are almost absent[11]. This might also critically contribute to the reduced migratory potential of CD fistula CLPF, since the ED-A subunit is usually increased during wound healing and is an important inducer of fibroblast migration[12]. A further stimulator of CLPF migration in the intestine is galectin-3 which is secreted by colonic epithelial cells[13,14]. Though galectin-3 is able to induce the migration of CLPF derived from CD fistulae, its expression is clearly decreased in CD fistulae[14]. These observations might explain the reduced mesenchyme-mediated wound healing potential in patients with CD fistulae. Other mechanisms have to step in to repair the epithelial barrier. Increased IEC migration is a mechanism aiming to replace the malfunctioning fibroblast migration, to improve wound repair and to restore intestinal barrier function. Thus, the migration of epithelial towards the defect might be induced as part of a compensatory mechanism, since the migratory potential of CD fistula CLPF is critically impaired[15].

A KEY ROLE FOR EPITHELIAL TO MESENCHYMAL TRANSITION

In order to migrate, IEC must undergo a conversion into mesenchymal-like cells so-called myofibroblasts. This process is called epithelial-to-mesenchymal transition. During EMT, differentiated IEC characterized by strong intercellular junctions and cell polarity lose their epithelial phenotype and acquire a mesenchymal differentiation featuring reduced cell-cell contacts and a fibroblast-like morphology and function[16,17]. EMT plays an important role during embryogenesis and organ development, but also for tissue remodelling and wound healing[16-18]. However, recent studies also suggested that EMT is critically involved in pathologic conditions, such as cancer growth and fibrosis[16,17]. While TGF is the most potent inducer of EMT *in vivo*[19], there are further markers for the onset of EMT, such as decreased expression of E-cadherin and β-catenin, translocation of membrane-bound-catenin into the cytosol or the nucleus and increased expression of β6-integrin[16,17,20,21].

Available data strongly suggest that EMT might also be critically involved in the formation of the TC layer covering the fistula tracts of CD patients. TGF-1 and TGF-2 expression are both upregulated in fistula lining cells as compared to regular IEC[22]. E-cadherin is involved in the formation of intercellular zonulae adherentes and is found at the lateral cell membrane at the cell-cell contact sites of normal IEC. In the TCs lining the CD fistula tract not only a decreased expression, but also redistribution of membranous E-cadherin is detectable. This change in localization of E-cadherin can especially be observed in the transition zone[22]. In TC-catenin expression is diffuse, much weaker and found in the cytoplasm and nuclei whereas it is located in the lateral cell membrane of normal mucosal IEC[22]. Six integrin expression normally is restricted to epithelial cells during embryonic development and organogenesis. Its re-induction indicates an important role of this molecule during intestinal EMT[20,21]. In contrast to normal IEC, TC localised in the transitional zones feature a strong staining pattern for 6-integrin[22]. IEC as well as mesenchymal-like myofibroblast-like TC expressed both of the epithelial markers, cytokeratin (CK) 8 and CK 20[22]. These staining patterns of the TC strongly suggest an epithelial origin of these myofibroblast-like cells that show characteristic features of EMT.

A further event that is characteristic to EMT, is the nuclear expression of the transcription factors SNAIL1 and SLUG (SNAIL2) which are activated by TGF and are involved in the down-regulation of E-cadherin[16,23,24]. Interestingly, there are different expression pattern for SNAIL1 and SLUG in CD fistulae. While SNAIL1 is heavily expressed in the nuclei of the TC lining the fistula tracts indicating transcriptionally active protein, SLUG is mainly detected in cells around the fistula tract and only to a very limited level in the fistula tract lining TC[25]. CLPF from CD patients with fistulae express higher SLUG mRNA levels than CLPF from patients without fistulae[26]. Expression of fibroblast growth factors 1 and 2 that are also associated with increased SNAIL1 expression, reduced E-cadherin expression and the onset of EMT are detectable in the tissue layers below the fistula tract and in the fistula-associated inflammatory infiltrates[25]. A number of EMT characteristic events are detectable in and around fistula tracts clearly supporting a role for EMT in the pathogenesis of CD-associated fistulae. The detection of epithelial markers in submucosal myofibroblast-like cells further supports this hypothesis and demonstrates the transformation of former IEC into these mesenchymal-like cells.

INVOLVEMENT OF MATRIX REMODELLING MOLECULES

The intercellular matrix is constantly remodelled by a number of enzymes that degrade all components of the extracellular matrix (ECM), namely the matrix metalloproteinases (MMP). Increased MMP activity finally results in immune-mediated tissue injury and is associated with a number of pathologies, such as cancer growth and CD[27-29]. The importance of MMPs for the development of CD is highlighted by the fact that in the murine DSS-induced colitis model, targeted deletion of MMP-9 has a protective effect[30,31] while mice overexpressing MMP-9 in the intestinal epithelium develop more severe colitis when compared to wild type animals[32]. Further, addition of MMP-3 caused extensive tissue injury in an *ex vivo* human fetal model of intestinal inflammation and tissue injury was effectively blocked by inhibiting MMP activity[33]. The physiological inhibitors of MMPs are the tissue inhibitors of MMP (TIMP) which are also secreted by the MMP producing cells[27].

In CD fistulae, strong MMP-3 expression is observed independent of the stage of inflammation. MMP3 mRNA and protein expression is detected in mononuclear cells and fibroblasts[34]. Inactive and active MMP-9 is expressed around CD fistulae and mRNA and protein levels are found in granulocytes and fibroblasts[34,35]. The activated isoform of MMP-13 is present in the supernatant of untreated CD fistula CLPF, but is almost absent in the supernatant of non-fistula CLPF. MMP-13 protein expression also is clearly detectable in mononuclear cells around CD fistulae[26,35]. In contrast, expression of MMP-1 and MMP-7 is only weak around CD fistulae, MMP-10 is not detectable and MMP-2 protein is equally expressed in fistula and control tissue. Activated MMP-2 is only detectable in CD fistulae[34]. Protein levels of TIMP-1, TIMP-2 and TIMP-3 are low around CD fistulae[34]. These observations suggest a critical role for matrix remodelling enzymes in fistula pathogenesis.

MOLECULES ASSOCIATED WITH CELL MIGRATION AND INVASION

The published data on fistula pathogenesis strongly sug-

gest that fistula originate from an epithelial injury and due to defective wound healing mechanisms IEC re-differentiate into mesenchymal-like cells acquiring migratory potential. In line with this assumption, molecules associated with IEC migration, such as 6-integrin, Ets-1 transcription factor and the Wnt-inhibitor Dickkopf-homolog 1 (DKK-1) are detected in CD fistulae.

While regular mucosal epithelial cells do not express 6-integrin, TC located in the transitional zone are strongly positive for β6-integrin staining[22] and fistula CLPF express higher 6-integrin mRNA levels than CLPF from control or CD patients without fistulae[26]. These observations are of particular interest, since 6-integrin expression has been associated with migration, invasion, metastasis and shortened survival in certain carcinomas, such as colorectal cancer, head-and-neck cancer, breast cancer or squamous cell cancer[20,36-39]. Up-regulated β6-integrin is associated with increased levels of EMT[20,38-41], represents a receptor for fibronectin and tenascin what might be important for cell migration, regulates secretion and activation of MMP-9[42,43] and mediates TGF activation and adhesion what has also been implicated in increased survival, progression and metastases of various tumours[44,45]. Additionally, 6-integrin can induce its own expression in an autocrine manner[46]. On a transcriptional level, 6-integrin is regulated by Ets-1 transcription factor[20]. While tissue samples from control and IBD-patients in remission display only low expression levels of Ets-1 protein, a strong staining signal is detected in tissue samples derived from patients with active inflammation and in TC along the fistula tract[47] providing further support for the regulatory effect of Ets-1 on 6-integrin expression.

The Wnt-inhibitor DKK-1 represents an important factor involved in the regulation of cell migration. Loss of DKK-1 has been associated with progression of certain types of carcinomas, such as CRC[48]. The secreted glycoprotein is capable to block IEC migration, is a potent antagonist of the canonical Wnt/β-catenin signalling pathway and has been implicated to act as a mediator of inflammation[49,50]. In the intestinal tissue of non-IBD control patients, Dkk-1 staining is very weak, while tissue samples from CD-patients with a perianal fistulae reveal strong DKK-1 staining in TC lining the fistula tracts and patients with active CD also exhibit considerable DKK-1 expression in inflammatory infiltrates[51].

TGF

TGF is the most important inducer of EMT[19] and expression of TGF-1 and TGF-2 is higher in TC than in normal IEC[22]. TGF induces the mRNA expression of interleukin-13 (IL-13) in the HT-29 IEC spheroid model of EMT and the secretion of IL-13 from fistula CLPF, but not from CLPF from non-IBD control patients or CD patients without fistulae[26]. The effect of TGF on IL-13 expression in IEC is mediated *via* -catenin and DKK-1[51]. In HT-29 IEC, TGF treatment induces DKK-1 levels and this effect is inhibited by knock-down of -catenin. Interestingly, knock-down of either-catenin or DKK-1 prevents the TGF-induced increase in IL-13 expression[51]. These observations fit to the nuclear staining pattern of -catenin in TC. Increased TGF levels in TC induce nuclear accumulation, meaning transcriptional activation, of -catenin what results in enhanced expression of DKK-1 and IL-13. The TGF-induced up-regulation of DKK-1 might serve hereby as a negative feed-back mechanism to control TGF-mediated effects. However, IL-13 decreases the expression of DKK-1 in IEC and fistula CLPF[51] what finally dis-ruptures this regulatory mechanism and might result in uncontrolled secretion of IL-13.

IL-13

IL-13 has been implicated in the pathogenesis of tissue fibrosis, such as in the lung or liver, and, in this context, induces the secretion of TGF[52-54]. It is mainly secreted by Th2-cells and its alpha 1 receptor (IL-13R1) is the signal transducing receptor while the alpha 2 receptor (IL-13R2) acts as a decoy receptor[55,56]. In TC lining the fistula tracts as well as in deformed crypts adjacent to the fistula, IL-13 and IL-13R1 are heavily expressed, while they are almost absent in the intestine of non-IBD patients, UC patients and CD patients without fistula regardless their inflammation status[26]. These observations suggest that IL-13 expression and associated effects might be induced in CLPF and fistula-associated IEC in a positive feedback mechanism. On a functional level, IL-13 induces the expression of SLUG and 6-integrin in HT29 cells grown as monolayers or spheroids. Interestingly the IL-13 induced 6-integrin expression is mediated, at least in part, *via* SLUG transcription factor and SLUG expression is sensitive to anti-IL-13 antibody treatment. However, in contrast to TGF, IL-13 treatment is not sufficient to induce EMT in the HT29-IEC spheroid model[26].

TNF

TNF has been demonstrated to induce EMT in IEC and is able to induce the expression of TGF[47,57,58]. Similar to IL-13, strong staining for TNF and its receptor, TNF-receptor I (TNF-RI) is detected in TC lining the fistula tracts as well as in IEC of adjacent crypts in CD patients. TNF is also expressed in fistula surrounding immune cells[25]. This observation further supports the hypothesis that TNF, similar to IL-13, induces its expression in an autocrine manner. Correlating, TNF induces its own expression in IEC and fistula CLPF *in vitro*[47,57]. In IEC and CLPF, TNF stimulates the expression of 6-integrin and Ets-1 transcription factor and knock-down of Ets-1 results in diminished 6-integrin levels in response to TNF. Of note, while TNF induces TGF and EMT in IEC, it is not sufficient to stimulate IL-13 neither in IEC nor in fistula CLPF[47]. These observations suggest that

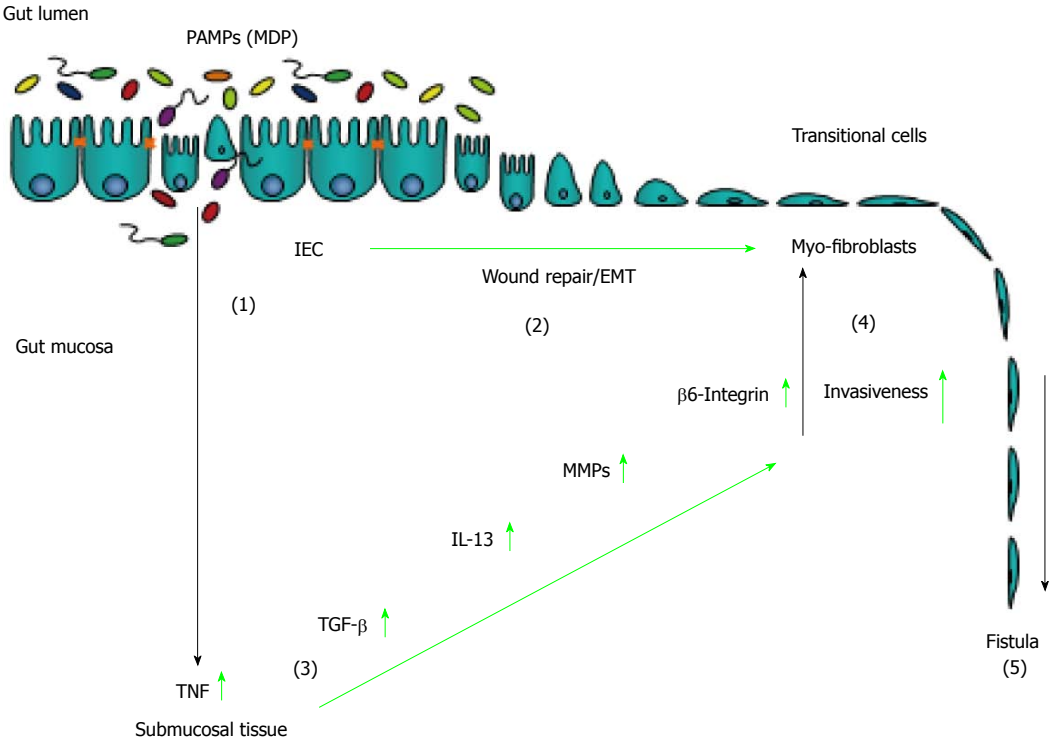

Figure 1 Pathogenesis of Crohn's disease-associated fistulae. An epithelial barrier defect favours the invasion of pathogen-associated pattern (PAMPs) into the gut mucosa (1). On the one hand, for wound healing purposes, intestinal epithelial cells undergo epithelial-to-mesenchymal transition (2). On the other hand, presence of PAMPs induced an inflammatory reaction resulting in increased secretion of TNF (3). TNF is able to induce secretion of TGF as well as to induce EMT and expression of molecules associated with cell invasiveness, such as 6-integrin. TGF-induced IL-13 and elevated activation of matrix remodelling MMPs critically contribute to invasive cell growth (4). Finally, EMT, MMP over-activation and elevated expression of invasive molecules contribute to the development of fistulae (5).

TNF, which is naturally present in acutely and chronically inflamed intestinal tissue, acts *via* two different pathways: TNF induces EMT on the one hand by its own, on the other had *via* TGF, as part of a wound healing mechanism. However, aberrant responses of IEC and/or CLPF to TGF then result in increased expression of IL-13. IL-13, similar to TNF, then stimulates its own expression *via* a positive feedback mechanism what finally causes the expression of molecules being associated with cell migration and invasiveness, such as Ets-1 and 6-integrin. TNF-induced effects can be effectively blocked by administration of an anti-TNF antibody *in vitro*[47], what might also explain, at least in part, the beneficial effect of anti-TNF antibodies for fistula treatment in CD patients.

PATHOGEN-ASSOCIATED MOLECULAR PATTERN

Polymorphisms within the nucleotide oligomerization domain 2 (NOD2) gene are associated with a fistulizing disease course of CD[59]. The bacterial wall component and pathogen-associated molecular pattern (PAMP), muramyl-dipeptide, is the natural ligand for NOD2 and following activation of NOD2, immune cells produce pro-inflammatory cytokines, such as TNF[60]. MDP treatment induces the expression of genes being associated with EMT as well as with cell invasiveness, such as TGF, SNAIL1, IL-13 and 6-Integrin, in IEC. While in non-fistula CLPF, MDP significantly induced mRNA expression of Ets-1, 6-Integrin, TNF, SNAIL1 and TGF, in fistula CLPF MDP treatment only induces mRNA levels of Ets-1 and TGF[47]. Since fistula CLPF express high levels of TNF and IL-13 *via* an autocrine mechanism, it might be that exogenous stimulation of these cells, *i.e.*, by MDP, is not sufficient to further induce TNF or IL-13 levels in these cells. Interestingly, lipopolysaccharide (LPS) does not induce any of the fistula-associated molecules in either IEC or CLPF pointing towards a specific role for the MDP-NOD2 axis in fistula pathogenesis. These observations suggest that distinct PAMPs might play a critical role for fistula pathogenesis by inducing EMT and genes being associated with EMT and cell invasiveness what makes the use of antibiotics in fistula treatment plausible.

CONCLUSION

Taken together, available data demonstrate that CD-associated fistulae originate from an epithelial defect that occurs during chronic inflammation. Having undergone EMT, IEC penetrate into deeper tissue layers causing tissue damage and a connection to other organs or the body surface. EMT of IEC is part of a wound repair mechanism as inflammation causes ongoing tissue damage and conventional wound healing mechanisms, such

as migration of fibroblasts, are impaired. The expression of EMT-associated molecules results in enhanced activation of matrix remodelling enzymes such as MMP-3 and MMP-9 causing further tissue damage and inflammation. Finally, soluble mediators such as TNF and IL-13 promote their own expression in an autocrine manner and enhance expression of molecules being associated with cell invasiveness. Subsequently, fistula formation and "growth" is constantly promoted and further supported by the presence of EMT-inducers, such as TGF, and PAMPs (Figure 1). Though current knowledge suggests a number of therapeutic options, new and more effective therapeutic approaches are urgently needed for patients suffering from CD-associated fistulae.

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TOPIC HIGHLIGHT

WJGP 5th Anniversary Special Issues (6): Crohn's disease

Escherichia coli in chronic inflammatory bowel diseases: An update on adherent invasive *Escherichia coli* pathogenicity

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Abstract

Escherichia coli (*E. coli*), and particularly the adherent invasive *E. coli* (AIEC) pathotype, has been increasingly implicated in the ethiopathogenesis of Crohn's disease (CD). *E. coli* strains with similar pathogenic features to AIEC have been associated with other intestinal disorders such as ulcerative colitis, colorectal cancer, and coeliac disease, but AIEC prevalence in these diseases remains largely unexplored. Since AIEC was described one decade ago, substantial progress has been made in deciphering its mechanisms of pathogenicity. However, the molecular bases that characterize the phenotypic properties of this pathotype are still not well resolved. A review of studies focused on *E. coli* populations in inflammatory bowel disease (IBD) is presented here and we discuss about the putative role of this species on each IBD subtype. Given the relevance of AIEC in CD pathogenesis, we present the latest research findings concerning AIEC host-microbe interactions and pathogenicity. We also review the existing data regarding the prevalence and abundance of AIEC in CD and its association with other intestinal diseases from humans and animals, in order to discuss the AIEC disease- and host-specificity. Finally, we highlight the fact that dietary components frequently found in industrialized countries may enhance AIEC colonization in the gut, which merits further investigation and the implementation of preventative measures.

Key words: Adherent invasive *Escherichia coli*; Inflammatory bowel disease; Crohn's disease; Pathogenesis; Epidemiology

Core tip: In this review we critically revise the findings on *Escherichia coli* (*E. coli*) populations associated with Crohn's disease and ulcerative colitis. Then we focus on adherent invasive *E. coli* (AIEC), especially in its mechanisms of pathogenicity and epidemiology. We discuss about AIEC disease- and host-specificity and we underline the importance of discovering specific molecular tools to detect AIEC for further epidemiologic studies. Finally we point out to a putative role of diet on AIEC gut colonization.

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ESCHERICHIA COLI IN INFLAMMATORY BOWEL DISEASE

The intestinal microbiota has been implicated in the pathogenesis of Crohn's disease (CD) and ulcerative colitis (UC), the main idiopathic inflammatory bowel diseases (IBDs)[1]. CD patients demonstrate an altered

intestinal microbial community, and the dysbioses present in colonic CD and ileal CD are different[2]. In contrast, a specific dysbiosis in UC is starting to be defined, although differences between studies have hampered attempts to reach a clear consensus to date[2-5]. A number of culture-based and molecular-based studies support the theory that *Escherichia coli* (*E. coli*) is a microbiological factor implicated in CD, but some controversy exists regarding its role in UC[2,6-17]. In this section, we examine data on *E. coli* populations in CD and UC related to abundance, association with disease activity, translocation of the gut mucosa, and pathogenic features of the strains to highlight the evidence that supports or refutes putative implications for this bacterium in each IBD subtype.

Abundance in the intestinal mucosa and correlation with disease activity

Several independent studies based on quantitative Polymerase Chain Reaction (PCR) have indicated that *E. coli* is augmented in CD patients in comparison with controls[2,6,11,13]. However, differences are especially significant for CD patients with ileal disease, and no clear association with colonic or ileocolonic CD has been demonstrated. On average, in our cohort, *E. coli* 16S rRNA gene copies accounted for 14% and 33% of total bacteria 16S rRNA gene copies in healthy subjects and ileal CD patients, respectively ($P < 0.001$)[13]. Of note, a higher abundance of *E. coli* was observed in active CD patients than in patients in remission[6,11,18]. Accordingly, a previous study using Fluorescent In Situ Hybridization (FISH) demonstrated increased *E. coli* numbers in the epithelium and within the lamina propria in active CD patients compared to inactive CD patients[14]. In addition, we determined that higher numbers of this species correlated with a reduced amount of time before relapse[11]. These findings are in agreement with previous data reporting that the higher numbers of *E. coli* isolated from the neoterminal ileum of CD patients are associated with early recurrence of the disease[7], and that high levels of antibodies against the *E. coli* outer membrane protein C (OmpC) correlate with disease progression, longer duration, and greater need for surgery among CD patients[19-21].

There is substantial controversy regarding the abundance of *E. coli* in the colonic mucosa of UC patients (Table 1). Several works have consistently reported no increase with respect to healthy subjects[2,6,7,11-13], arguing against a putative role for *E. coli* in UC, while others have reported increased *E. coli* abundance in UC patients[8,10,14,16,18,22,23]. As in the majority of these studies both CD and UC patients were analyzed, these controversial observations can not be explained by differences in methodology between studies. We postulate that they can be attributable to differences in the disease severity of the patients included in the studies, as increased numbers of *E. coli* have been associated with activity status in UC patients. Using FISH, epithelium-associated *E. coli* were found to be more abundant in active UC compared to inactive UC or controls[14], and quantitative PCR indicated that increased numbers of *E. coli* were present in active UC patients compared to inactive UC patients[22] as well as in inflamed *vs* non-inflamed UC tissue[23].

Altogether, substantial evidence supports an overgrowth of *E. coli* in ileal CD patients, while there is still no convincing data that exists for other IBD subtypes. Further studies aimed at comparing the abundance of *E. coli* in IBD patients categorized by disease subtype and assessing any correlation with activity status of the disease would shed light on the role of this bacterium in each IBD subtype and its putative application as a diagnostic and/or prognostic tool.

E. coli localization in the intestinal mucosa

E. coli has been found in the mucus layer, close to the intestinal epithelial cells and in ulcers of both CD and UC patients[24,25]. Translocation of the intestinal mucosa has been primarily observed in CD[6] and higher amounts of intracellular *E. coli* were detected in inflamed compared to non-inflamed mucosa[6,26]. With FISH and immunohistochemistry, *E. coli* has been detected scattered within the lamina propria, either in the extracellular space or inside macrophages, as well as in the subserosal layer, the perivascular areas of the submucosa, the muscle layer, and in germinal centers of lymph follicles of CD patients[8,14,27]. A recent study using high throughput sequencing indicated a greater proportion of *E. coli* reads in the lymph nodes of ileal CD patients than other CD patients[28]. Interestingly, *E. coli* DNA was also more frequently found in the granulomas of CD patients (80%) than in non-CD control patients (10%) in a study that used Laser Capture Microdissection and PCR[29]. In contrast, *E. coli* has not been frequently found to translocate the mucosa of UC patients[8,24,25], although some controversy exists as some authors have detected *E. coli* in the lamina propria of UC patients[14,27].

The majority of the aforementioned studies are based on techniques that do not distinguish viable bacteria from dead bacteria. Further studies should study the viability of translocated *E. coli*, particularly in lymph nodes and granulomas, as these locations would be more relevant to establish a link between this bacterium and CD pathogenesis. These studies should also focus on UC patients to clarify the existing controversial data. A lack of *E. coli* translocation in UC would suggest that *E. coli* does not play a primary role in UC pathogenesis or that it plays a different role than in CD.

Pathogenic features of the strains

E. coli strains isolated from IBD patients are clonally diverse[6,13,17] and belong to distinct serotypes[6,13,30] and to different sequence types[6,31-33]. Although a close genetic relationship was detected in a study of IBD pediatric patients[34], the hypothesis that there is a particular clone associated with IBD has largely been ruled out.

In turn, *E. coli* strains isolated from IBD patients

Table 1 Controversy about *Escherichia coli* imbalances in ulcerative colitis

Ref.	Method	Samples	Comments
Increased *E. coli* abundance in CD but not UC			
Martin *et al*[12]	Culture	Biopsies	Specially hemagglutinin-positive strains
Martinez-Medina *et al*[13]	qPCR	Biopsies	Specially in ileal CD
Lopez-Siles *et al*[11]	qPCR	Biopsies	Specially in active CD
Darfeuille-Michaud *et al*[7]1	culture	Biopsies	Specially in ileal lesions
Baumgart *et al*[6]1	qPCR	Biopsies	Specially in ileal CD
Willing *et al*[2]1	qPCR	Biopsies	Specially in ileal CD
Increased *E. coli* abundance in CD and UC			
Mylonaki *et al*[14]	FISH	Biopsies	Specially in active UC patients
Kotlowski *et al*[10]	culture	Biopsies	
Rehman *et al*[16]	cloning	Biopsies	
Fujita *et al*[8]	qPCR	Biopsies	
Schwiertz *et al*[18]	qPCR	Feces	Specially in active CD patients
Sha *et al*[22]	qPCR	Feces	Specially in active UC and CD patients
Pilarczyk-Zurek *et al*[23]2	qPCR	Biopsies	Specially in inflamed UC tissue

[1]Increased *E. coli* abundance in CD with respect to controls but UC patients were not included in the study; [2]Increased *E. coli* abundance in UC with respect to controls but CD patients were not included in the study. CD: Crohn's disease; UC: Ulcerative colitis; *E. coli*: *Escherichia coli*.

primarily belong to the B2 and D phylogroups in conjunction with extraintestinal pathogenic *E. coli* (ExPEC). Some works demonstrate major colonization by B2+D phylogroups in IBD patients in comparison with healthy controls[10,31], but in other studies, a similar distribution of phylogroups exist between IBD and healthy subjects[13,29,30,33-36]. Differences between studies could be based on the types of samples analyzed, as it has been reported in healthy individuals that transient *E. coli* (more likely to be found in feces) are principally A and B1, whereas resident *E. coli* (more likely to be found in the mucosa) mainly belong to the B2 and D phylogroups[35]. Therefore, studies based on mucosal samples tend to indicate enrichment of B2 and D strains, even in healthy controls. Another factor that could influence the distribution of phylogroups in IBD is the disease severity of patients analyzed, as an increased proportion of B2 and D isolates has been found in active IBD patients[32], which was significantly associated with the inflammation state of IBD tissues[30]. This denotes a shift in *E. coli* populations to isolates that are better adapted to the inflamed tissue in IBD and/or that are involved in the inflammation itself. Of note, no differences in phylogroup distribution between CD and UC have ever been reported.

E. coli isolated from IBD patients carry different sets of virulence genes that are characteristic of ExPEC strains, whereas intestinal pathogenic *E. coli* are rare or absent[6,10,13,30,32,34,36-39]. These virulence factors are also frequent in *E. coli* from healthy subjects and are considered "colonization factors" necessary for successful establishment in the intestinal mucosa[40]. Virulence gene profiles are inexorably linked with the phylogenetic origins of the strains. Based on the distribution of phylogenetic groups, virulence-associated genes characteristic of ExPEC were more frequently found in IBD patients than in healthy subjects in those studies where B2+D predominated in IBD[10,31], whereas no differences were found in other works[13,36,37,41,42]. A shift in the phylogroup distribution would then lead to an increased proportion of *E. coli* equipped with colonization factors that would facilitate establishment and persistence in IBD patients. However, it is not clear whether this shift occurs specifically in IBD patients or is a general trend taking place in industrialized countries[43]. Although no particular genetic traits distinguish *E. coli* from the intestinal mucosa of CD or UC, some virulence factors have been found to be differentially distributed between these IBDs. For example, a diarrhea-associated hemolytic *E. coli* strain called cell-detaching *E. coli* (CDEC), which commonly harbors hemolysin, cytotoxic necrotizing factor 1, pilus P and S-fimbria genes, was found in 24% of UC *E. coli* and only in 4.7% of CD *E. coli*[44]. The gene *usp* encoding for the uropathogenic-specific protein was also more frequently found in UC *E. coli* than in CD *E. coli*[30]. Recently, *E. coli* carrying the *iroN* gene, which encodes for a receptor for iron-chelating siderophores, was more frequently isolated from inflammatory and unchanged mucosa of active-phase UC patients[23].

On the other hand, approximately one decade ago, Darfeuille-Michaud *et al*[45] discovered a new pathotype of *E. coli* with distinctive phenotypic pathogenic traits that was associated with CD, but not with UC, named adherent invasive *E. coli* (AIEC). Altogether, these observations suggest that specific *E. coli* types could be involved in each IBD. We further discuss this issue in the section dedicated to AIEC prevalence in ulcerative colitis.

ADHERENT INVASIVE *E. COLI*

To date, AIEC is the most likely candidate to cause specific damage to people who are genetically susceptible to the development of CD, and therefore the following sections will focus on discussing the most recent research findings on this pathovar. We review (1) the

latest research regarding AIEC pathogenicity; (2) the prevalence and abundance of the pathotype in several intestinal disorders, discussing its putative contribution to other intestinal diseases in addition to CD; (3) the evidence that supports a lack of host-specificity and thus a risk for zoonosis; and (4) recent research that points to a putative role for environmental factors in the fate of AIEC development in the intestine.

Definition

The AIEC pathotype was defined as *E. coli* strains that (1) are able to adhere to differentiated Caco-2 and/or undifferentiated I-407 intestinal epithelial cells with an adhesion index equal or superior to 1 bacteria per cell; (2) are able to invade I-407 cells with an invasion index equal or superior to 0.1% of the original inoculum; (3) involve host cell actin polymerization and microtubule recruitment in bacterial uptake; (4) do not have known invasive determinants; and (5) are able to survive and replicate within J774-A1 macrophages[45]. Since its definition, invasive determinants characteristic from ExPEC have been detected in some AIEC, but not consistently in all AIEC, and thus are not a particularity of the AIEC pathotype[6,13,36,46-48].

Molecular basis of AIEC pathogenicity

Pathogenicity mechanisms of AIEC have mainly been studied in the reference AIEC strain LF82, and its features have been comprehensively linked to many characteristics of CD pathogenesis.

Adhesion to intestinal epithelial cells is in part mediated by type 1 pili, which interact with the glycoprotein CEACAM6 in a mannose-associated manner[49,50]. CEACAM6 is overexpressed in CD patients with ileal disease, which makes them more susceptible to overcolonization by AIEC. Although type 1 pili is present in almost all *E. coli*, including non-pathogenic strains, we have recently demonstrated that AIEC strains usually present FimH adhesin variants that allow them to more efficiently bind intestinal epithelial cells[31]. Some non-AIEC strains carry these mutations as well, but they do not express type 1 pili. Flagella are also important for adhesion to and invasion of intestinal epithelial cells and elicit the secretion of the pro-inflammatory cytokine IL-8 and chemokine CCL20 in polarized intestinal epithelial cells, which in turn leads to the recruitment of macrophages and dendritic cells to the site of infection[51,52]. The further secretion of INFγ and TNFα by macrophages and lymphocytes leads to CEACAM6 expression, which enhances AIEC colonization. The binding of LF82 type 1 pili to CEACAM6 and flagella to TLR5 in intestinal epithelial cells induces the production of HIF-1a and activation of the classical NF-κB pathway[53]. In turn, these molecules cooperatively control the transcription of IL-8 and pro-angiogenic factors contributing to inflammation and vascularization.

The intermediate filament vimentin, expressed on the host cell surface of mesenchymal cells, has been recently proposed to act as a receptor for AIEC[54]. At the intracellular side, vimentin leucine-rich repeats interact with NOD2 leading to the recruitment of these proteins at the plasma membrane. This is necessary for a proper function of NOD2 in terms of antigen detection, NF-κB activation and autophagy induction. CD patients have specific NOD2 variants (L1007fs and R702W) that are unable to interact with vimentin and, in turn, they localize in the cytosol. That leads to a defective inflammatory response, autophagy induction and handling of CD-associated AIEC. Altogether, NOD2 and vimentin appear to play an important role in AIEC recognition and polymorphisms in these two proteins may have an impact on the ability of AIEC to colonize the host.

A new host-microbe interaction that mediates adhesion of LF82 to intestinal epithelial cells and involves a bacterial and a human chitinase has recently been proposed[55]. Chitinases are enzymes that hydrolyze chitin, a long-chain polymer of an N-acetylglucosamine. The authors demonstrate that specific polymorphisms in two chitin binding domains characteristic of LF82 and other pathogenic *E. coli* are required to interact with an N-glycosylated asparagine of the human chitinase CHI3L1. Interestingly, human chitinases are overexpressed in intestinal epithelial cells and moderately expressed in cells of the lamina propria during inflammation.

Outer membrane vesicles (OMVs) containing the transmembrane protein OmpA play a role in LF82 invasion of intestinal epithelial cells[48]. OmpA binds the endoplasmic reticulum-localized stress response chaperone Gp96 that is overexpressed on the apical surface of ileal epithelial cells in patients with CD. OMVs fuse with host cells, and it is thought that the release of bacterial effectors that are still undefined is involved in the actin polymerization and microtubule recruitment that occurs during invasion. Point mutations in the *ompA* sequence of LF82 and other B2 strains mediate better interactions with Gp96[56]. In turn, Gp96 is overexpressed in the ileum of CD patients, which renders them more susceptible to AIEC infection.

Once inside the host cell, LF82 bacteria can be found in several types of intracellular compartments: individually or in groups within single membrane vacuoles, within damaged vacuoles, or within LC3-positive autophagosomes, which indicates that autophagy restricts a subpopulation of intracellular LF82 bacteria[57]. Nevertheless, it was recently demonstrated that AIEC can abrogate the autophagic process[58]. Intracellular LF82 activates NF-κB, leading to the increased expression of MIR30C and MIR130A in T84 cells and in mouse enterocytes, and the upregulation of these microRNAs reduces levels of ATG5 and ATG16L1, inhibiting autophagy and enhancing the inflammatory response. In turn, defects in autophagy mechanisms related to the ATG16L1 and IRGM genes have been associated with CD patients, and these defects confer an advantage for AIEC to survive inside human cells[57]. Therefore, it is a combination of host deficiency factors and AIEC

pathogenicity that determines the fate of intracellular *E. coli* survival.

In addition to adhesion and invasion capacity, LF82 is also able of translocating *via* the M cells of the Peyer's patches, gaining access to the lamina propria. This interaction is mediated by type 1 pili and long polar fimbriae (Lpf), which can interact independently with GP2, a surface protein specific to M cells. It is of note that the sites of initial inflammation in CD are the Peyer's patches and colonic lymphoid follicles; thus, this mechanism of translocation is consistent with early clinical signs of the disease[59].

Another mechanism that can facilitate bacterial translocation is the ability of LF82 to alter intestinal permeability by inducing the expression of the pore-forming protein claudin-2[60] and by displacing ZO-1 and E-cadherin from apical tight junctions, leading to decreased transepithelial resistance and loss of barrier function[17,61]. Besides, pro-inflammatory cytokines like TNFα can drive alterations in intestinal permeability[62]. As AIEC infection induces the secretion of large amounts of TNFα and IL-8[17]; thus, the loss of barrier function induced by LF82 can in part be mediated by the induction of TNFα secretion.

A novel mechanism of pathogenicity observed in LF82 and two other AIEC strains (O83:H1 and UM146) is the evasion of host immune responses *via* subversion of the IFNγ pathway in intestinal epithelial cells[63]. Phosphorylation of the Signal Transducer and Activator of Transcription STAT-1 is blocked, thus preventing the transcription of IFNγ-dependent genes, which reduces host immune responses and results in an inability to mount an appropriate anti-microbiocidal response. Enterohemorrhagic *E. coli* (EHEC) strain O157:H7, in part through its Shiga toxin, is also able to block tyrosine phosphorylation and activation of STAT1 after IFNγ stimulation, in contrast with enteropathogenic *E. coli* E2348/69 or commensal *E. coli* HB101 which do not present this mechanism of pathogenicity. However, AIEC do not present Shiga toxins. Presumably a small secreted peptide may be responsible for this pathogenic mechanism in AIEC[63].

Once AIEC has gained access to the lamina propria, these bacteria can be engulfed by macrophages. Intramacrophage LF82 do not escape into the cytoplasm but induce the formation of a large vacuole (phagosome) that fuses with lysosomes[64], suggesting that AIEC bacteria have the ability to replicate in an environment with acidic pH, oxidative stress, active proteolytic enzymes, and antimicrobial compounds. Indeed, it was demonstrated *in vitro* that an acidic environment is necessary for replication of AIEC LF82 bacteria[64]. The protease HtrA and the thiol-disulfide oxidoreductase DsbA have been reported to be important for survival and replication within macrophages[65,66]. The authors linked these proteins to the ability of LF82 to resist the stress conditions of the phagolysosomes, as isogenic mutants for these proteins were less efficient in growing in acidic and nutrient-poor medium, and these proteins were overexpressed not only in LF82 during macrophage infection but also in acidic nutrient-poor medium. Interestingly, the overexpression of HtrA is dependent on the LF82 background, as non-pathogenic *E. coli* do not overexpress that protein under similar growth conditions. The RNA-binding protein Hfq, which functions as a global posttranscriptional regulator of gene expression, has also been implicated in survival and replication within macrophages and in stress tolerance but also other aspects of LF82 pathogenicity, such as adhesion and invasion capability[67]. Hfq binds small regulatory RNA molecules, facilitating their interaction with mRNA, but the target genes are still unknown.

Continuous replication of LF82 within macrophages results in the secretion of high levels of TNFα without inducing host cell death[68]. This can explain inflammation and granuloma formation in the gut of CD patients, which has been demonstrated *in vitro*[50,69,70]. A direct role for LF82 in delaying apoptosis of infected macrophages and dendritic cells has recently been reported[71]. LF82 infection was found to alter the function of caspase-3, a protease that plays an essential role in apoptosis, and to increase degradation of this molecule in the proteasome.

Also supporting AIEC capability to replicate within immune cells, strain LF82 was able to replicate within monocytes isolated from CD patients for the first 20 h after infection but then CD monocytes started to clear intracellular bacteria[72]. Interestingly, those patients with polymorphisms in *CARD15* gene (R702W, G908R and 1007fs) showed reduced early inflammatory response towards AIEC infection with decreased levels of IL-1β, IL-6 and IL-10. In contrast, Asp299Gly mutation in TLR4 had no effect on monocyte response to AIEC. Besides, a recent study revealed that CD monocyte-derived dendritic cells stimulated with lipopolysaccharide show an attenuated inflammatory response with decreased levels of IL-6 and IL-1β, as well as an impaired autophagy with reduced LC3 expression[73]. Moreover, these cells had a reduced capacity to support the expansion of allogeneic Th17 cells from CD4+ memory T cells. The authors propose that mucosal Th17 activation in CD patients is a secondary event in response of poor bacterial clearance due to defects in innate immunity. Further studies showing AIEC effects on CD defective dendritic cells regarding, not only cytokine release, but also autophagy function and the level of IL-17A response induction in T cells, are necessary to decipher whether the alterations observed in lipopolysaccharide-stimulated dendritic cells equally occur after AIEC exposure.

AIEC LF82 bacteria are also able to invade and replicate within human neutrophils, but in contrast to its behavior inside macrophages and intestinal epithelial cells, LF82 induces the autophagic death of infected neutrophils, which later undergo an alternative cell death process called NETosis[74]. In neutrophils, LF82 are localized inside autolysosomes, as observed by the colocalization of phagosome and lysosome markers, but there is no

acidification, which suggests that LF82 avoids autolysosome maturation. Infected neutrophils secrete cytokines, in particular IL-8, contributing to mucosal inflammation.

The ability to form biofilms is a pathogenic feature frequently found among AIEC strains. We found that 17 out of 27 AIEC strains and only 9 out of 38 intestinal non-AIEC strains were biofilm producers[75]. Motility and flagellar type are of relevance in biofilm production, as non-motile strains were not able to form biofilms, and all strains with the H1 flagellar antigen were strong biofilm producers. Recently, Chassaing *et al*[76] have demonstrated the ability of the LF82 strain to form biofilms on intestinal epithelial cells using cell culture and animal models.

Genetic factors characteristic of the AIEC pathotype

Despite all the research conducted on AIEC pathogenicity, we still do not know the genetic factors that are characteristic of the AIEC pathotype. The majority of genes related to its pathogenicity are not AIEC-specific, as is the case for *fimH*, *ompA*, *dsbA* or *btrA,* and are present in the majority of *E. coli* strains, including non-pathogenic strains[31,48,65,66]. Point mutations or differential gene expression are involved in the increased fitness and/or virulence of AIEC strains. Unfortunately, these genetic factors have been studied in very few strains or exclusively in the prototype strain LF82. Conversely, virulence genes that are not usually present in non-pathogenic *E. coli*, such as *afaC*, *pks* or *lpf*, have been found frequently, but not consistently, in AIEC strains[13,59,77]. AIEC strains are clonally diverse, belong to different serotypes and carry different sets of virulence genes that are characteristic of ExPEC strains; these features also describe non-AIEC ExPEC-like strains inhabiting the intestinal mucosa[13]. The AIEC pathotype comprises high genotype variability, which complicates the identification of specific genetic factors of the pathotype.

It is of note that despite the genetic similarity between AIEC and ExPEC, the latter generally does not exhibit the AIEC phenotype. We determined that only 4 out of 63 ExPEC strains of different origins were AIEC-like[78], conferring a particular identity on the pathotype. Identification of additional genetic elements or the differential expression of key genes that must be involved in AIEC pathogenicity represents an important milestone that can be achieved through genome- and transcriptome-based studies.

Four AIEC genomes belonging to B2 strains have been sequenced and published to date[46,47,79,80], and comparative genomics have been carried out for the LF82 and NRG857c strains[47]. Although novel virulence factors not previously found in AIEC by PCR genotyping, such as a type-6 secretion system, have been detected in genomic islands of the sequenced strains, genomic studies have corroborated the notion that AIEC resembles ExPEC. Unique sequences for AIEC were found in common between the LF82 and NRG857 strains. However, both strains belong to the same phylogroup and serotype (B2 O83:H1), which indicates they are genetically very close. Given the high variability of AIEC seropathotypes, studying the distribution of these genes in other AIEC strains is essential to confirm whether these elements are common features of the pathotype or are strain-specific. Comparative genomics of phylogenetically distant AIEC strains would presumably reveal a significantly greater number of genetic differences. Although it will complicate the situation, sequencing additional AIEC strains from different phylogenetic origins is crucial to determine the common genetic features involved in the AIEC phenotype.

AIEC localization in the intestinal mucosa

AIEC have generally been isolated from tissue samples, but there is no evidence regarding its exact localization within the intestinal mucosa. Although AIEC are invasive bacteria, they have not been convincingly observed within intestinal epithelial cells or in the lamina propria in resected tissue or mucosal biopsies. The studies conducted by Martin *et al*[12] and Eliott *et al*[36] addressed whether *E. coli* are intraepithelial or mucosa-associated by treating biopsies with gentamicin. This approach has brought indirect evidence of *E. coli* invasion of intestinal epithelial cells in CD but not in UC. However, the complete AIEC phenotype was not studied in the intracellular *E. coli* strains obtained from these studies.

Currently, identifying the exact localization of AIEC strains in the mucosa is nearly impossible to do, as no molecular tools that specifically target the AIEC pathovar are available. Some evidence has been obtained using animal models infected with known reference strains. For example, by staining for the O83 antigen, it has recently been demonstrated that the LF82 and NRG857c strains colonize the ileum, cecum and colon of several mouse models and that they are located at the base of the crypts and within goblet cells[81]. Engineered LF82 with a plasmid containing the GFP protein permitted fluorescence-microscopic examination of the localization of LF82 in the nematode *C. elegans*. In this situation, there was robust gut colonization, but bacteria remained in the lumen and were not attached to intestinal epithelial cells[67]. To visualize the extent of bacterial adhesion and invasion in *in vivo* infection, Low *et al*[55] stained *E. coli* lipopolysaccharides with specific antibodies and compared basal levels of fluorescence in uninfected mice (corresponding to indigenous bacteria) with levels in infected mice. They found higher counts of stained bacteria in the intestinal epithelial cells and lamina propria of infected mice, suggesting AIEC intestinal epithelial cell invasion and translocation.

A pathobiont rather than a true pathogen

Despite the virulence genes that are encoded in the genome of many AIEC strains and the mechanisms of pathogenicity reported for the prototype strain LF82, AIEC are generally considered pathobionts. This assumption is supported by the fact that, although at a

lower frequency than in CD, healthy subjects can carry AIEC in their intestinal mucosa[6,13,17,45,82]. The prevalence varies between studies, ranging from the absence to 15.8% of colonic samples with AIEC and from 6.2% to 18% in ileal samples. Although AIEC bacteria may colonize the intestinal mucosa of non-IBD patients, these bacteria usually do not translocate a healthy mucosal barrier, as bacterial invasion of the mucosa has not frequently been observed in control patients[14] and intracellular *E. coli* was rarely cultivated from the colonic mucosa of healthy subjects (from the absence to 9%)[6,26,83]. AIEC strains are more abundant, and consequently more frequently found, in the ileum than in the colon of healthy subjects. We found that AIEC accounted for 3.58% and 0.95%, respectively, of the ileal and colonic *E. coli* populations[13]. Accordingly, a larger number of AIEC LF82 bacteria were attached to ileal than to colon tissue in *ex vivo* samples from healthy subjects infected with this AIEC strain[84]. Altogether, these data suggest that AIEC can more easily colonize the ileum with respect to other *E. coli*, and at least approximately 1 out of 6 healthy individuals can be considered "asymptomatic carriers".

Genetic or environment-derived host defects at the intestinal barrier may determine the ability of AIEC to colonize and translocate the gut. A number of host deficiencies frequently found in CD patients have been linked with the increased ability of AIEC LF82 to cause infection. For example, these defects include the overexpression of the CEACAM6 and Gp96 receptors in the apical membrane of intestinal epithelial cells, which facilitates AIEC adhesion and invasion[48,49], or defects in autophagy related to NOD2, ATG16L1 and IRGM function and expression, which impair the ability of host cells to resolve infections[57,85]. Additionally, it has been suggested that the altered bile salts metabolism in CD patients could enhance the expression of long polar fimbriae in AIEC, which could permit better translocation *via* M cells[86]. Moreover, decreased levels of the protease meprin, which are characteristic of severe inflammation in IBD patients, have been proposed to determine the fate of AIEC in terms of their ability to colonize the host, as these proteases degrade type 1 pili[87].

PREVALENCE AND ABUNDANCE OF AIEC IN IBD

CD

Intraepithelial *E. coli* with adherent and invasive properties were isolated from the sigmoid colon mucosa in 29% of CD patients[12] and in 90% of CD patients in a cohort composed of ileal, ileocolonic and colonic disease phenotypes[36]. Differences between studies could be explained by the disease activity status of the cohort of patients, who were mainly in the relapse stage in the latter study.

In the last decade, several independent laboratories have reported a higher prevalence of AIEC in CD patients than in healthy subjects[6,13,17,45,82]. Unfortunately, not all of these studies categorized CD patients by their disease subtype or analyzed prevalence based on anatomic location in the gut. The first study was conducted by Darfeuille-Michaud *et al*[45] in 2004 and revealed that 22% of CD patients with ileal involvement harbored AIEC strains in ileal chronic lesions and at a similar frequency in healthy mucosa. However, AIEC bacteria were more likely to be found in the early ileal lesions that occurred in patients after ileostomy (36.4%). AIEC strains were only isolated from the colon of 3.7% of CD patients with a colonic disease phenotype. The authors proposed an association between AIEC and ileal CD and suggested that the pathovar could be involved in the initiation of the inflammatory process. Conversely, Baumgart *et al*[6] reported a prevalence of AIEC strains in the ileum of 38.5% of CD patients with ileal involvement and 37.5% with colonic CD, indicating that AIEC is associated both with ileal and colonic disease phenotypes. Sasaki *et al*[17] demonstrated that 24.3% of CD patients exhibited AIEC strains, but neither the localization of these strains in the gut nor the disease phenotypes of the positive patients were detailed. A similar prevalence was reported by Dogan *et al*[82] in the ileum of CD patients with ileal disease. We detected AIEC strains at a higher frequency in comparison with previous studies, most likely due to the methodological approach used. Whereas other studies analyzed from 1 to 15 *E. coli* colonies per patient, we searched for AIEC strains in a collection of 95 - 150 *E. coli* colonies per patient. This approach not only enabled us to obtain a more accurate prevalence value but also to study the abundance of AIEC strains within the *E. coli* population. We detected AIEC strains in the ileum of 54.5% of CD patients and in the colon of 50% of CD patients[13]. Although data depicted by disease subtype were not reported in the original work, we also found a higher prevalence in CD patients with ileal involvement (66.7% of ileal and 58.3% of colonic samples) than those with colonic disease (50% of ileal and 25% of colonic samples). Colonic CD patients denoted also a high prevalence of AIEC, what supports the observations of Baumgart *et al*[6], but the pathotype was more frequently found in the ileum than in the colon of CD patients, in line with the findings of Darfeuille-Michaud *et al*[45] The abundance of AIEC, defined as the percentage of AIEC within the *E. coli* population, was low and variable, ranging from 1% to 50%. On average, AIEC isolates represented 9.3%, 3.7% and 3.1% of *E. coli* isolates in ileal, ileocolonic and colonic CD patients, respectively. Jensen *et al*[84] supported these data using quantitative PCR targeting indigenous LF82 bacteria. The increased expression of CEACAM6 in the ileum of ileal CD patients may explain the higher prevalence and abundance of AIEC in CD patients with ileal involvement. However, additional host-microbial interactions or environmental factors may be involved in colonization of the colonic mucosa, as no differences

in CEACAM6 expression exist at the level of the colon between CD patients and control subjects[49]. Our work demonstrates that AIEC are more prevalent than expected in all CD disease subtypes, reinforces the hypothesis that the microenvironment of ileal CD specifically favors AIEC expansion, and suggests that the colon is also a niche effectively colonized by AIEC.

UC

More than two decades ago, the adhesion capabilities of *E. coli* from both UC and CD patients were assessed. Mannose-resistant adhesion was characteristic of *E. coli* from both IBDs, which raised the question of whether adhesive *E. coli* could also be involved in UC pathogenesis[88,89]. Recent studies have confirmed that, in UC patients, adherent *E. coli* strains are found as frequently as[90] or even more frequently than[34,91] in CD patients. An undefined adhesion pattern was most prevalent in *E. coli* from both UC and CD patients[42], although aggregative adherence was particularly frequent in UC patients[42,90]. Molecular tools to detect adhesive determinants of IBD *E. coli* did not demonstrate specific adhesion factors in UC *E. coli* in comparison to CD *E. coli*[10,37,41,42], whereas in other studies UC *E. coli* carried some adhesion factors more frequently than CD *E. coli*[30,34,44]. Some of these studies are based on pediatric or newly diagnosed patients, which provides supporting arguments for the early contribution of adherent *E. coli* to IBD rather than being its development a consequence of inflammation. Moreover, the higher frequency of *E. coli* B2 strains with at least one positive adhesion-related gene was correlated with disease activity in UC patients (86% in active *vs.* 13% in non-active patents)[32]. Therefore, there is substantial agreement among studies regarding the adhesion capacity of *E. coli* strains from UC patients.

Intracellular *E. coli* were cultured from 47%[36] and 19%[12] of UC patients in two studies using gentamicin protection assay. However, few works have sought to identify the AIEC pathovar in UC patients, and some controversial results have been obtained. In the first study that searched for AIEC in UC any of UC patients had AIEC bacteria in their colon[45], and similar results were obtained in a later study[92]. In contrast, in studies with larger cohorts, one of them based on pediatric patients, AIEC were detected in 7.2% to 10% of UC patients[17,93]. Other investigators that studied the invasion ability of IBD *E. coli*, but did not study the complete AIEC phenotype, detected a high prevalence of invasive strains in UC patients (37.5%)[44]. Moreover, similar invasion rates in I407 cells were observed for *E. coli* from pediatric UC and CD patients[42], whereas in a previous study the invasion index using differentiated Caco-2 cells was lower in *E. coli* from UC than CD patients[17]. Noteworthy, the intra-macrophage survival capacity of *E. coli* strains was found to be highest in UC patients from a cohort of newly diagnosed IBD patients. Unfortunately, no information about adhesion and invasion abilities was provided[30].

Sasaki *et al*[17] observed that although AIEC from UC were less invasive than CD *E. coli*, they induced the secretion of similar amounts of TNFα and higher amounts of IL-8, suggesting that UC-associated *E. coli* are distinct from those associated with CD. Accordingly, a recent study reported that CD *E. coli* are frequently *lpf*+ *afaC*+, whereas UC *E. coli* do not possess *lpf* gene and frequently harbor the *afaC* and *pks* genes together[77]. Lpf mediate translocation of bacteria via M cells, while the afimbrial adhesin AfaC mediates a diffuse adherence to and invasion of intestinal epithelial cells and also induces vascular endothelial growth factor expression. The polyketide synthase gene complex (*pks*) contains the genes to synthesize the metabolite colibactin, a genotoxin with the ability to cause epithelial DNA damage.

The evidence collected to date suggests that *E. coli* strains with adhesive and other virulence properties could be involved in UC pathogenesis, but further work clarifying the role of these strains in conjunction with host defects in the mucosal barrier is needed. Furthermore, in view of the few studies and conflicting results regarding AIEC prevalence in UC, additional studies characterizing *E. coli* populations from different anatomical sites, and for both affected and unaffected tissue, in active and inactive UC patients would be of relevance to elucidate the possible role of AIEC in UC.

E. COLI POPULATIONS IN OTHER INTESTINAL DISEASES: IS AIEC INVOLVED?

Colorectal cancer

An analysis of fecal bacterial diversity by pyrosequencing demonstrated that the *Escherichia/Shigella* genus was enriched in colorectal cancer (CRC) patients[94]. In contrast, studies conducting quantitative PCR did not find an increase in the *E. coli* population in CRC[8,91]. However, intracellular *E. coli* has frequently been found in CRC patients. Swidsinski and collaborators detected intracellular *E. coli* in 87% of patients with CRC and not in controls using a gentamicin protection assay[95]. Similarly, Martin *et al*[12] isolated intramucosal *E. coli* from 33% of tumors in CRC patients and 9% of control subjects, surpassing the prevalence found among IBD patients, and Bonnet *et al*[96] isolated intramucosal *E. coli* in 86% of colon cancer tumor specimens and 48% of diverticulosis samples. Moreover, high levels of mucosa-associated *E. coli* correlated with poor colorectal carcinoma prognostic factors and a higher proliferative index of epithelial cells, suggesting a role for these bacteria in tumor progression.

E. coli strains isolated from the study by Prorok-Hamon *et al*[77] were hemagglutination-positive, adherent to HT29 and I407 intestinal epithelial cells and frequently able to invade I407 cells, all characteristics that resemble the AIEC pathotype. A recent study conducted by the same research group showed that at least one of the isolates obtained from a patient with CRC shared the com-

plete AIEC phenotype. In addition, *E. coli* isolated from a pediatric cohort with polyposis, who were included as a healthy control group, showed the highest invasion efficiency compared with *E. coli* strains isolated from IBD children[42]. However, as far as we know, there is no data regarding the prevalence of AIEC in patients with CRC.

Several studies have demonstrated that *E. coli* associated with CRC are frequently colibactin-producing[72,93-95]. Not only is the *pks* genomic island encoding for the genotoxin colibactin frequent in CRC, but other cyclomodulins such as CNF, CDT and CIF. Buc *et al*[97] found that cyclomodulin-encoding genes were over-represented among *E. coli* from CRC patients (65.8%), particularly distal colon cancer (76.5%), compared with diverticulosis samples (19.54%). These molecules can be genotoxic and/or modulate cellular differentiation, apoptosis, and proliferation. Prorok-Hamon *et al*[77] observed that CRC *E. coli* frequently harbored the *pks* gene but also the adhesins AfaC and LpfA, partially resembling those *E. coli* isolated from CD and UC. These factors confer the ability to adhere to and invade I407 cells, to upregulate vascular endothelial growth factor expression in intestinal epithelial cells, and presumably, to translocate *via* M cells and cause genotoxicity to host cells. Recently, pathogenic cyclomodulin-positive *E. coli* strains were found to be more prevalent in the mucosa of patients with advanced stages of the disease[96].

Few studies have been focused on *E. coli* populations in CRC patients do date, and the results obtained point to a putative role for a subset of *E. coli* with pathogenic features relevant to CRC pathogenesis. Given that AIEC possessing virulence factors relevant to enterocyte adhesion and invasion, vascular endothelial growth factor expression and carcinogenesis have been detected in CRC patients and the fact that intramucosal *E. coli* with features similar to AIEC have been more frequently found in CRC than in IBD patients, further studies determining the prevalence of AIEC in CRC are needed to corroborate or refute the hypothesis for a putative role for AIEC in CRC.

Coeliac disease

Coeliac disease is a chronic inflammatory disorder exclusively affecting the small intestine, in which genetically predisposed individuals feature a permanent intolerance to dietary gluten. Several studies have provided evidence that coeliac patients exhibit intestinal microbial dysbiosis, similar to what occurs in IBD patients. In a study based on PCR-TGGE of duodenal samples, *E. coli* was found more frequently in coeliac children (92.1%) than in healthy children (20%)[98]. Quantification of *E. coli* by FISH showed also that this species was more abundant in active coeliac patients than in inactive patients and controls[99], but this was not observed in fecal samples[100]. Another study found changes in *Enterobacteriaceae* diversity and increased virulence-gene carriage in *E. coli* isolates from coeliac children[101]. In particular, *E. coli* strains largely belonging to the B2 and D phylogenetic groups and carrying ExPEC-like features, *e.g.*, pilus P and hemolysin A, were found to be more abundant in celiac patients when compared to healthy controls. This dysbiosis of the *E. coli* population is similar to that found in CD patients.

Given the association between *E. coli* and coeliac disease in terms of abundance and the correlation with disease activity, as well as the genetic similarities between isolates from the intestinal mucosa of coeliac patients and CD patients, further studies aimed at identifying the AIEC phenotype amongst coeliac *E. coli* isolates are of interest to better define the disease specificity of the AIEC pathotype.

ADHERENT-INVASIVE *E. COLI* IN ANIMALS WITH INTESTINAL DISEASE

AIEC strains isolated from CD patients genetically resemble avian pathogenic *E. coli* and other animal ExPEC. We studied the AIEC phenotype in a strain collection obtained from animals with extraintestinal infection and intestinal disease to determine the disease and host specificity of the AIEC pathotype. All these strains were classified as ExPEC in terms of their phylogenetic origin and virulence genotype. ExPEC strains of extraintestinal origin rarely shared the AIEC phenotype, whereas ExPEC-like strains of intestinal origin were frequently AIEC-like in cats (82%), dogs (35%) and swine (32%) with intestinal disease[102]. The high prevalence of AIEC in companion and farm animals highlights a putative risk of zoonosis between humans and animals. In a previous study, Simpson *et al*[103] detected AIEC in boxer dogs. Interestingly, these dogs suffered from granulomatous colitis, a disease with pathological features that overlap with CD, which supports the role of AIEC in human CD and analogous diseases in animals.

Altogether, these results suggest that the AIEC pathotype is disease-specific rather than host-specific and raises the question of whether there is a possible route of transmission between animals and humans. Further studies examining the distribution of AIEC strains in different healthy and diseased animals and in the environment would contribute to our understanding of the epidemiology, putative reservoirs, host-specificity and possible routes of transmission of AIEC.

ENVIRONMENTAL FACTORS INVOLVED IN THE SUCCESSFUL COLONIZATION OF AIEC

Recent studies have implicated some emulsifiers and food stabilizers frequently used in developed countries as having a role in AIEC colonization. Maltodextrin, a polysaccharide derived from starch hydrolysis that is used as food additive, has been shown to markedly enhance AIEC biofilm formation and adhesion to intestinal epithelial cells and macrophages[104]. Maltodextrin fa-

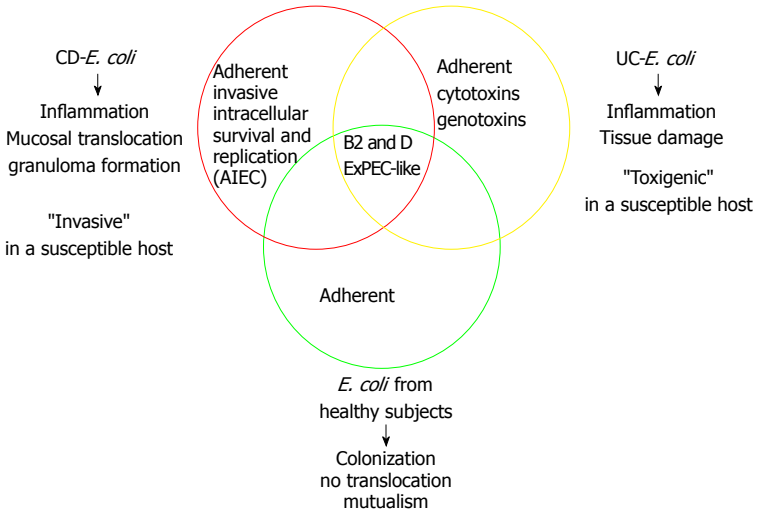

Figure 1 Features of inflammatory bowel disease-associated *Escherichia coli* and impact of this species on Crohn's disease and ulcerative colitis.

vors type 1 pili expression, which is required for biofilm formation and adhesion. Moreover, a higher prevalence of the gene *malX*, which is essential for maltodextrin metabolism, was found in bacteria isolated from ileal CD patients than from healthy controls (71% *vs* 18%, respectively). These observations suggest that a diet rich in maltodextrin would aid maltodextrin-utilizing bacteria, would enhance *E. coli* gut colonization, and thus contribute to dysbiosis. Furthermore, polysorbate-80, an emulsifier commonly used in processed foods, was found to enhance translocation of the AIEC HM605 strain across M cells and intestinal epithelial cells[105]. Using animal models, we also observed that a diet enriched in fat and sugar induced dysbiosis and low grade-inflammation[106]. In this work we also showed that dysbiosis and low-grade inflammation in susceptible individuals lead to increased AIEC colonization, what in turn exacerbated the inflammatory response and epithelial barrier disruption.

The type of dietary fiber intake may influence bile acid metabolism. For example, daily dietary supplementation for four weeks with the purified fiber components pectin and cellulose in humans leads to differential bile acid composition. In cellulose-treated volunteers, cholic acid increased whereas deoxycholic acid decreased, which inversely occurred in pectin-treated individuals[107]. Increased concentrations of cholic acid and chenodeoxycholic acid have been reported in CD patients[108], and lithocholic acid has been reported particularly in ileal CD patients[109]. Interestingly, all of these bile salts induced the expression of the *lpf* operon in AIEC LF82 strain[86]. Therefore, dietary fiber consumption could also influence the tropism of AIEC for CD ileal tissue by altering bile acid composition and thus the expression of *lpf* in AIEC in the gut.

These studies demonstrate that dietary components may impact the success of AIEC in colonizing the host and therefore contribute to disease susceptibility. For that reason, intervention studies are needed to evaluate the effects of diet, probiotics, and/or prebiotics on the intestinal microbial community, including the AIEC population with respect to CD activity status and disease progression.

AIEC, A CAUSE AND A CONSEQUENCE OF INFLAMMATION

Several studies based on animal models have shown that there is a need of microbial dysbiosis and/or intestinal inflammation to succeed with AIEC infection. An effective colonization only occurs in mice that have been treated with antibiotics[50,81,110], dextran sodium sulfate[69] or high-fat/high-sugar diet[106] before infection, having these treatments an effect on gut bacteria composition and mucosal homeostasis. Moreover, Craven *et al*[111], nicely showed that moderate to severe ileitis produced by protozoan infection in mice models induced dysbiosis and proliferation of endogenous mucosally invasive *E. coli*. These works suggest that inflammation and dysbiosis favors AIEC proliferation. Therefore, AIEC overgrowth in the intestine can be seen as a consequence of inflammation.

On the other hand, it has been recently shown that AIEC infection itself induced lasting changes in the intestinal microbiota[112]. This study was conducted on mice lacking flagellin receptor TLR5 (T5KO) which are prone to develope spontaneous colitis. The authors hypothesized that transient colonization of T5KO mice by AIEC results in an altered gut microbiota community with greater proinflammatory potential, which can persist in the host and induce chronic inflammation due to its increased levels of lipopolysaccharide and flagellin. The effects of AIEC infection on host mucosal immunity, barrier integrity and inflammation induction have been demonstrated in multiple animal models[50,60,69,81,106,110] but the work of Chassaing *et al*[112] is the first showing that AIEC infection contribute to intestinal dysbiosis. Overall, these studies suggest that AIEC overgrowth in the intestine can be seen as a cause of inflammation.

Therefore, inflammation can instigate imbalances in *E. coli*, especially the AIEC pathotype and, in turn, these bacteria can be involved in a further dysbiosis and in-

creased intestinal inflammation.

CONCLUSION

Substantial evidence indicates that *E. coli* is involved in CD and growing data suggest that this species is also a contributing factor in UC pathogenesis (Figure 1). Studies focused on defining virulence gene profiles of *E. coli* populations have shown that *E. coli* associated to the mucosa of healthy subjects resemble those of IBD patients. Genes related with adhesion, iron transport, capsule formation and toxins are present in *E. coli* from both healthy subjects and IBD patients. These features are thought to be necessary for an effective colonization of the intestinal tract. However, the intestinal microenvironment in IBD patients, especially those in relapse, would predispose to *E. coli* proliferation. Moreover, *E. coli* from CD patients have probably evolved towards the AIEC pathotype, which has the capacity to adhere to and to invade intestinal epithelial cells, as well as to survive and replicate within a number of cell types. Virulence properties of AIEC described to date can explain several features of CD pathophysiology such as inflammation, mucosal bacterial translocation and granuloma formation. Conversely, *E. coli* strains from UC patients appear to present a "toxigenic" behavior rather than the "invasive" pathogenic mechanism of CD- *E. coli*. Recent research has pointed out that *E. coli* from UC patients frequently carry virulence genes related to cytotoxicity and genotoxicity, which can contribute to mucosal inflammation and tissue damage. This is in accordance with previous works that did not found *E. coli* translocating the epithelial barrier of UC patients, and could be linked with some aspects of UC pathophysiology.

Since the AIEC pathotype was defined one decade ago, substantial research has been conducted focusing on the identification of the mechanisms of pathogenicity and also in the field of epidemiology with regard to CD. However, additional epidemiologic studies are still needed to corroborate the role of AIEC in CD and to clarify the AIEC disease- and host-specificity. An important limitation to epidemiological studies is the absence of specific molecular tools to detect and quantify this pathotype, as the current available techniques to identify the AIEC pathotype are based exclusively on phenotypic screening of cultured bacteria, which is highly time-consuming. The execution of large-scale epidemiologic studies would also provide new insights into its distribution, putative reservoirs and transmission pathways. Moreover, the molecular bases of AIEC pathogenicity are still not fully understood, as only a few model strains have been studied and there is a wide variety of serotypes and phylotypes within the AIEC pathotype. Genomic and transcriptomic studies including wider and more diverse AIEC strain collections could assist in identifying new genetic elements associated with the AIEC phenotype, which may help us to gain a better understanding of the mechanisms of pathogenicity and could result in significant advances in the detection of new therapeutic targets for CD.

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TOPIC HIGHLIGHT

WJGP 5th Anniversary Special Issues (6): Crohn's disease

Similarities and differences between Behçet's disease and Crohn's disease

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Abstract

Behçet's disease (BD) is a chronic inflammatory condition with multisystem involvement. Approximately 10%-15% of patients present with gastrointestinal involvement. Involved sites and the endoscopic view usually resemble Crohn's disease (CD). In addition to intestinal involvement, oral mucosa, the eyes, skin, and joints are commonly affected. No pathognomonic laboratory test is available for the diagnosis of either disease. Management approaches are also similar in various aspects. Differentiating BD from CD is highly challenging. In this article, the similarities and differences between BD and CD in terms of epidemiology, etiopathogenesis, clinical and imaging findings, and histopathological and therapeutic approaches are reviewed.

Key words: Behçet's disease; Crohn's disease

Core tip: Behçet's disease and Crohn's disease are chronic inflammatory conditions caused by lesions similar to those seen in the bowels. There are similar and different clinical findings, however both diseases show intestinal inflammation. The differential diagnosis may be difficult when the symptoms of the two disease processes are very similar. This review focuses on the similar and different characteristics of Behçet's disease and Crohn's disease.

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INTRODUCTION

Behçet's disease (BD), which was first defined by Hulusi Behçet, a Turkish dermatologist, in 1937, is a chronic inflammatory disease with multisystem involvement[1]. It presents with remission and exacerbation of mucocutaneous, ocular, articular, vascular, or gastrointestinal lesions. Crohn's disease (CD), on the other hand, is a chronic relapsing inflammatory disorder of the gastrointestinal tract, presenting with BD-like extra-intestinal manifestations[2]. Both of these chronic immune-mediated inflammatory disorders are likely to affect patients at a younger age accompanied by fluctuating courses. It is possible that a patient with CD meets the criteria for BD. The differential diagnosis in some cases is quite difficult, particularly in the presence of gastrointestinal involvement. Differentiation is usually based on the involvement of different organs. This review aims to investigate the similar and different characteristics of BD and CD.

EPIDEMIOLOGY

The prevalence of BD varies geographically and the

disease is more prevalent in certain groups. It is most common in populations clustered along the ancient Silk Road. Turkey has the highest prevalence (80-370 cases/100000), followed by Asia and the Middle Eastern countries, including Israel, Saudi Arabia and Iran. BD can be seen in all countries worldwide due to immigration[3-6]. The age at onset of the disease is usually between 20.8 and 40 years, as it is more common in young individuals[3]. Patients aged 16 years with initial symptoms, considered as childhood-onset BD, have also been reported. The male-to-female ratio varies regionally. The disease is more common among men in Russia, Saudi Arabia, Iraq, Lebanon, Jordan, Kuwait, Greece, Italy, Turkey, and Iran, while it is more frequent among women in Japan, South Korea, and Israel[3,7].

The incidence of CD may also vary regionally. The incidence of the disease is highest in the United Kingdom, North America, and the northern part of Europe. The prevalence of CD was found to be 133/100000 in the state of Minnesota, United States in 1991. In recent years, several studies showing an increasing incidence ratio have been reported. The incidence is highest in young individuals aged 15 to 29 years. The prevalence of the disease is similar in men and women (the male/female ratio is 2.9-0.76/1)[8-14].

GENETIC FACTORS

There are familial BD cases in the literature, suggesting that genetic factors play a role in the pathogenesis of the disease. The ratio of familial cases is between 0 and 18.2%. The genetic association between the HLA-B51 gene and BD was first reported in 1982 by Ohno[15]. This association has been confirmed in many different ethnic groups. The HLA-B5 gene, particularly the HLA-B5101 allele gene, may be a strong candidate locus responsible for the development of BD and HLA-B51 itself may be the major disease susceptibility gene for BD[16]. It is more likely that the HLA-B51 gene is directly *involved* in the *hyperactivity* of *neutrophils*. Increased neutrophil function has also been reported in HLA-B51-positive BD patients[17,18].

Familial aggregations and a high degree of disease concordance in twins with CD have been recognized for quite some time. The concordance rate has been reported to be 3% in dizygotic twins and up to 35% in monozygotic twins[2]. Recent studies have provided an insight into genetic disorders responsible for susceptibility of the disease. Furthermore, these studies have strengthened the evidence that major cytokines, cytokine receptors and cell types are involved in the underlying pathogenesis of the disease. Nucleotide oligomerization domain 2 (NOD2) is the major susceptibility gene for CD. Genome-wide association studies have demonstrated a number of susceptibility genes where NOD2 is encoded. The nucleotide oligomerization domain 2 gene is located at the CD susceptibility locus on chromosome 16q12[19,20].

PATHOGENESIS

Immunosuppressive agents, which are used in the management of autoimmune disorders, are highly effective in BD, and the role of autoimmunity has been widely discussed in the pathogenesis of the disease[21,22]. However, anti-nuclear antibody (ANA) positivity, anti-Ro, and anti-La antibodies, which are usually found in autoimmune disorders, have not been found in BD. Several studies have demonstrated the presence of anti-endothelial antibodies, anti-lymphocytic antibodies, and heat-shock protein 60 (HSP60) in BD; however, these antibodies have not been strongly associated with the disease[23]. Additionally, major histocompatibility complex (MHC) Class Ⅱ molecules have been associated with autoimmunity. However, BD is strongly associated with HLA-B5, a MHC Class I antigen.

BD is likely to be an autoinflammatory disease, as it presents with mucocutaneous lesions and episodic arthritis without deformity with a very strong acute phase response during these episodes. In BD, neutrophils are implicated in the inflammatory process of natural immune system-mediated disease (caspase pathway, IL-1, IL-18) similar to autoinflammatory diseases[21]. Mediterranean fever (MEFV) gene mutations, which are the main causes of familial Mediterranean fever (FMF), an autoinflammatory disease, are frequently found in BD[24,25]. However, the presence of clinical manifestations including uveitis, vasculitis, and thrombosis, which are not seen in autoinflammatory diseases, and the absence of serositis, a very common pathology in autoinflammatory diseases, does not suggest its autoinflammatory nature. Thus, currently, BD is considered to be neither an autoinflammatory nor an autoimmune disorder[21].

Furthermore, large and small vessel vasculitides may be present in BD. Thrombotic occlusions of the venous branches and aneurysm formations in the arterial vessels may develop. Arterial involvement may lead to bleeding and organ failure, and ultimately death. Immunosuppressive therapies can be effective in the resolution of vasculitis[26]. Vasculitis-related alterations have been observed in biopsy specimens of oral aphthae, genital ulcers, and skin lesions[27]. As vasculitis is considered to be a major component involved in the pathogenesis of BD, it is recommended that the disease should be evaluated under systemic vasculitides[28].

Several microorganisms of the oral microbial flora have been indicated in the pathogenesis of BD[29,30]. Atypical streptococcal colonization is increased in the oral mucosa. A hyperimmune activity against *Streptococci* has been shown in various studies. *Streptococcus sanguinis* causes increased interleukin-6 (IL-6) and interferon gamma (IFN-γ) secretions in the peripheral blood T-cells[31]. *Escherichia coli* and *Staphylococcaceae* species have been reported to increase inflammatory cytokines in BD patients. There are also studies showing regression of BD lesions with antibiotherapy in the literature[32].

Microorganisms that are involved in normal colon

microflora with a mutual relationship with the immune system are also considered to play a role in the underlying pathogenesis of CD. Several products that are produced by these microorganisms such as butyrate and propionate contribute to intestinal inflammation, by affecting immune system cells and cytokines in patients with genetic susceptibility to CD. Reduced mucin production in epithelial cells of the intestinal mucosa is another possible culprit. Genome-wide association studies have revealed a relationship between gene mutations in mucin expression (MUC1, MUC19 and PTGER4) and CD[2,19].

Innate immune system cells are mostly implicated in the immunopathogenesis of CD[2]. Pattern recognition receptors such as Toll-like receptors (TLR) and nucleotide binding domain (NOD) like receptors (NLR) have a critical role in the recognition of the molecular patterns of innate immune system cell pathogens. There is a strong association between NOD2/CARD15 polymorphisms and CD. NOD2/CARD15 encodes an intracellular receptor that is expressed predominantly in monocytes and Paneth cells. These pattern receptors are substantially expressed by dendritic cells lying beneath the intestinal epithelium. Dendritic cells may have reduced regulatory T-cell stimulation, which leads to immune tolerance in CD. These cells are responsible for organization of the relationship between microbial products and immune system cells, and identify immunity or tolerance development. The inflammasome complex of the lamina propria, which is implicated in mononuclear cells, is crucial for the immune response. The stimulation of NLPR3, caspase-1, and pro-interleukin-1 causes a significant increase in pro-inflammatory cytokines including tumor necrosis factor alpha (TNF-alpha) and interleukin (IL)-6. IL-17, IL-23, and IL-27 are crucial players in inflammatory alterations during the disease process[19,33]. Some authorities have adopted CD as an autoinflammatory disease due to its potent inflammation pathways[34]. Unlike patients with BD, the incidence of MEFV gene mutations remains unchanged in patients with CD[35]. The ANA and anti-neutrophil cytoplasmic antibodies (ANCA) positivity is higher in the patient population than healthy individuals. Nearly 40% to 70% of patients also have positive anti-*Saccharomyces cerevisiae* antibodies (ASCA), which are associated with disease severity. The ASCA positivity is higher in patients with CD than BD[36-38].

CLINICAL MANIFESTATIONS

Extra-intestinal manifestations

BD is a multisystem condition usually presenting with oral mucosa, ocular, articular, and vascular involvement. Gastrointestinal, neurological, and cardiac involvement are relatively infrequent. Nearly all patients suffer from recurrent oral ulcers. These ulcers are classified as large, small, or herpetiform, based on their size. They are extremely painful and may involve the buccal mucosa, labial mucosa, tongue, the soft and hard palate, and the pharynx. The incidence of genital ulcers with scar formation is relatively low compared with oral ulcers. These painful ulcers are quite similar to oral ulcers in appearance. They may be found in the scrotum and penis in men, and the vulva, vagina, and cervix in women. Additionally, nearly two-thirds of patients with BD have skin changes including acne-like papules, pustules, pseudofolliculitis, and erythema nodosum-like lesions. Due to superficial thrombophlebitis, shifting, and painful subcutaneous nodules can be palpable[7,39-43].

The pathergy phenomenon is a hyper-reactive response to minor trauma. The test is based on the principle of using a 21-25-gauge needle inserted into the skin. Positive test results show papulopustular lesions in the skin or erythematous reactions of the surrounding tissue within 24-48 h. The positive predictive value of the pathergy test varies regionally as the rate of positive pathergy test differs in different countries, and is highest in countries along the ancient Silk Road (30%-70%). The diagnostic value of diagnostic criteria is reduced when pathergy positivity is excluded. In addition, the pathergy test, which involves intradermal monosodium urate crystals, is more sensitive[7,43].

Ocular involvement in BD includes anterior or posterior uveitis, vitritis, retinal vasculitis, retinal vein thrombosis, corneal ulcers, and retrobulbar neuritis. Ocular disease may be the initial manifestation of the disease. BD-associated uveitis is defined as chronic and recurrent non-granulomatous panuveitis and retinal vasculitis with a bilateral course. The disease usually presents with acute inflammatory episodes that resolve within days or weeks. Recurrent episodes may result in permanent vision loss. Furthermore, as uveitis is rarely accompanied by conjunctivitis, scleritis, episcleritis, or sicca syndrome, other conditions should be suspected in patients with ocular involvement[40-44].

Musculoskeletal disorders are also common in patients with BD. Palindromic asymmetric arthritic exacerbations involving the knee, wrist, and ankle may develop. Chronic erosive arthritis is relatively rare. The incidence of sacroiliitis has been reported to increase in patients with BD. Due to peripheral arthritis characteristics and sacroiliac joint involvement, BD is evaluated in the spectrum of seronegative spondyloarthropathy. An overlap of relapsing polychondritis and BD, known as mouth and genital ulcers with inflamed cartilage (MAGIC) syndrome, may also develop in patients with cartilaginous inflammation[42-45].

BD-related vasculopathy differs from other vasculitides, due to its pattern of arterial and venous involvement. Venous thrombus may develop. It may present with superficial thrombophlebitis or involve deep veins, as well as the inferior/superior vena cava, the right atrium, or intracranial large sinuses. The major hepatobiliary disease is Budd-Chiari syndrome, which is one of the leading causes of mortality[46]. Unlike other thrombotic events, embolism is not anticipated. Primary thrombosis,

which is often accompanied by right atrial thrombi, may occur in the pulmonary artery and its thin branches. In addition, arterial aneurysms are common. Pulmonary artery aneurysms may lead to massive bleeding and a fatal outcome[40-42].

Moreover, central nervous system (CNS)-related symptoms may develop secondary to vascular events, such as sinus thrombus and intracranial aneurysms. Primary parenchymal involvement including meningitis and encephalitis, mostly in the pons and mesencephalon, is also seen in patients with BD. It is also known as Neuro-BD, accounting for 10% of patients. In addition, longitudinal extensive transverse myelitis (LETM), characterized by spinal cord lesions, may occur. Histopathological examination of neurological lesions typically shows an inflammatory cellular infiltration of the surrounding vessels[40,41].

CD is a complex disorder, which primarily involves the small intestine and the colon. However, various extra-intestinal manifestations of the disease including oral and genital ulcers, erythema nodosum, uveitis, and arthritis may also be observed[2]. Skin changes may be seen in 5%-10% of patients. Erythema nodosum (5.6%-13.5%), pyoderma gangrenosum (0.75%-0.15%), and acute neutrophilic dermatoses, also termed Sweet' s disease, are among the main skin lesions. Other skin conditions include oral aphthous lesions, perianal lesions, large ulcers, fissures, fistulas, and aseptic abscesses[47,48]. Pathergy positivity is extremely low in patients with CD, compared to those with BD[49].

The most common ocular conditions are uveitis, episcleritis, conjunctivitis, and blepharitis. Non-granulomatous anterior uveitis may develop and recurrent episodes may result in permanent vision loss. Ocular complications are not associated with disease severity. Additionally, retinal vasculitis, which is extremely rare, has been reported in the literature as case studies[47-51].

The clinical association between spondyloarthropathy and CD has been well-established. Nearly 10%-15% of CD patients are complicated by spondyloarthropathy. Both peripheral and axial arthropathies may be seen in CD. Peripheral arthropathies often present as asymmetric pauciarticular involvement. It is usually acute and self-limited, and the severity of the disease is reduced in parallel with decreased disease activity without sequelae. Persistent erosive monoarthritis has been described. Axial involvement resembles ankylosing spondylitis. Bilateral sacroiliitis, as well as spondylitis of the lumbar vertebrae and syndesmophytes may be seen. Chronic low back pain is the main symptom. It is frequent in asymptomatic sacroiliitis. Half of patients with CD have sacroiliac joint abnormalities, as evidenced by X-ray images[52,53].

There are several studies showing a 1.5- to 3.5-fold increase in the risk of venous thromboembolism in CD. Some authors have suggested that it can be attributed to increased hospitalization and surgical interventions. On the other hand, the risk of arterial aneurysm and thromboembolism remain unchanged. However, mesenteric ischemia may occur[54,55]. The incidence of Takayasu's arteritis has been reported to increase in patients with CD[56].

Primary sclerosing cholangitis is common in patients with CD, which has been reported in up to 10% of cases[48]. Although neurological signs of CD are not evident, neuroradiological imaging studies have demonstrated alterations in brain morphology[57].

Intestinal manifestations

Gastrointestinal manifestations are quite common in patients with BD. The most frequently observed signs include abdominal pain, diarrhea, nausea, anorexia, and abdominal distension. Despite the diffuse nature of the symptoms, ulcerations known as intestinal BD are relatively few. Gastrointestinal involvement varies regionally and according to the diagnostic method used. The incidence ranges from 15% to 50% based on symptoms alone and from 0.7% to 30% based on imaging or endoscopic findings[58]. Gastrointestinal involvement is higher in patients with childhood-onset BD[59]. BD-associated gastrointestinal involvement may affect all areas from the mouth to the anus. The terminal ileum and cecum are the main sites of ulcers, while few ulcers are seen in the esophagus and gastric duodenum. The most common site of involvement is in the segmental colon. Less than 15% of patients have diffuse intestinal involvement. The differentiation of intestinal BD from inflammatory bowel disease is sometimes quite challenging. The disease can be misdiagnosed as CD or ulcerative colitis during endoscopic examination. Fistulas, hemorrhage, and perforations mimicking CD may also be present. The shape of ulcers varies endoscopically from irregular to round and oval with a punched-out appearance, they are large (> 1 cm) and typically located in the deep layers. Longitudinal ulcers are rarely seen. The presence of less than six round and focal ulcers strongly indicate intestinal BD. Colonic ulcers include volcano-type and aphthous type lesions. Rectal and anal lesions are extremely rare[36,60-62].

Abdominal pain, diarrhea with or without bleeding, fatigue, weight loss, and fever are common manifestations of CD. Odynophagia, dysphagia, and dyspeptic symptoms are also seen in the case of esophageal and gastroduodenal involvement. Diarrhea is a common presentation, but often fluctuates over a long period of time. Fibrotic strictures may lead to repeated episodes of small bowel, or less commonly colonic, obstruction. Transmural bowel inflammation is associated with the development of sinus tracts, which may give rise to a fistula or abscess formation. Perianal disease, such as anal fissures, perirectal abscesses, and anorectal fistulas, occur in more than one-third of patients with CD[63]. CD may affect all areas from lips to the anus. Lesions were located in the terminal ileum in 40%-83%, colon in 32%, perianal region in 10%-15%, and the upper gastrointestinal tract in 4%[2,58,63]. Endoscopic findings of proximal CD include mucosal edema, focal and diffuse erythema, nodular lesions, erosion, and ulcers[64]. A diagnosis of CD should be considered in any patient who presents

with isolated terminal ileum involvement and ileoscopy should be performed in all patients. The earliest lesions in CD consist of tiny punched-out ulcers. Deeper ulcers can occur throughout the entire wall of the colon. Cobblestoning–Serpiginous and linear ulcers are seen along the longitudinal axis of the entire colon. CD lesions are discontinuous and can be adjacent to normal tissue. Rectal involvement is suggestive of ulcerative colitis rather than CD. In addition, perianal lesions are frequently seen in CD with fistula formation[36,65].

PATHOLOGY

In BD, neutrophilic infiltration, lymphocyte aggregation of the surrounding vessels, and vascular proliferation have been observed in biopsy specimens of oral apthae and genital ulcers. Neutrophil-predominating infiltration, abscess formation and vasculitis-related changes may be present in skin lesions. Aggregation of lymphocytes, neutrophils, and eosinophils as well as edema and leukocytoclasia occur in the pathergy test site within the first 12 h. In the presence of large vessel involvement such as aortic involvement, medial elastic fiber ruptures or loss may be seen, while proliferation of the vaso vasorum and lymphocytic infiltration of the surrounding tissue may develop. Lymphocytic and necrotizing vasculitides are other conditions involving pulmonary arteries, veins, and septal capillaries. In addition, transmural necrosis and aneurysms of great vessels and pulmonary arteries may arise. Despite the non-specific nature, perivascular lymphocyte/plasma cell infiltration and myelin loss of parenchymal CNS lesions may develop[26,27,66-68].

Furthermore, inflamed intestinal BD may lead to mesenteric vasculitis with ischemia or necrosis of the intestines. Ulcer specimens often show non-specific patterns, including fibrinopurulent exudates and necrotic debris in active ulcers and transmural fibrosis in chronic ulcers. Inflammation from the lumen to the serosa is present in the perforated site with mural necrosis. Vasculitic changes secondary to the inflamed surrounding tissue and thrombus formation in the small vessels including both arteries and veins are other critical manifestations. Lymphoid follicles may be seen due to mucosal erosion in some cases. The differential diagnosis of these lesions, which are histopathologically suggestive of CD is highly challenging[26,68].

Histopathological characteristics of CD are discontinuous cryptic architectural abnormalities, mucin preservation at active sites, discontinuous inflammation, focal cryptitis, and epithelioid granulomas. Granulomas in histological sections are key features of CD, but are not necessary for diagnosis. In the submucosa, fibromuscular obliteration, nerve fiber hyperplasia and transmural lymphoid aggregates are found. Transmucosal increases in lamina propria cellularity and neutrophils are an indicator of disease activity[69].

Both BD and CD may present with transmural enteritis and colitis. Longitudinal ulcers, cobblestone appearance, and anorectal fistula are usual findings in Crohn's colitis. The presence of granulomas in biopsy specimens indicates CD, while vasculitides are suggestive of BD[36].

DIAGNOSTIC CRITERIA

Although there is no specific diagnostic test for BD, diagnostic criteria sets described at different time points are available. The International Study Group (ISG) criteria[70], which were defined in 1990, are the most commonly used criteria for the diagnosis of BD (Table 1). These criteria are based on the most frequent clinical signs of BD. In addition, some cases of CD meet these criteria[71].

Several diagnostic and classification criteria for CD have been proposed[8,72-75] (Table 1). The location and appearance of lesions are important for the diagnosis of CD. According to the Vienna[74] and Montreal[75] classifications, the diagnosis of CD is established by three variables: (1) age at diagnosis; (2) disease location; and (3) behavior of the disease. The Lennard-Jones criteria are based on endoscopic, surgical/histopathological, radiological and clinical findings[73]. The Copenhagen criteria include histopathological confirmation of CD[8]. A diagnostic criteria set for CD based on alterations in gastrointestinal morphology was published in 2011[72]. However, no validated and widely adopted criteria set is currently available for the diagnosis of CD in clinical practice. The diagnosis usually relies on the patient history, physical examination, laboratory results, imaging studies, and endoscopic findings in combination with histopathological examination. Patients with BD, particularly with intestinal involvement, may be misdiagnosed and mismanaged as CD by clinicians with insufficient experience and knowledge on BD.

MANAGEMENT

As BD is a multisystem condition, effective management of the disease requires a multidisciplinary approach. Although the disease should be primarily managed by a rheumatologist, consultation is provided by a dermatologist, neurologist, gastroenterologist and cardiovascular surgeon, if necessary. The disease is inflammatory; therefore, immunosuppressive and immunomodulatory agents are first-line therapies. Due to the limited number of randomized-controlled clinical trials, management usually depends on the clinical experience of the treating physician. In 2008, the European League Against Rheumatism (EULAR) published a recommendation guideline for the management of BD[76].

The management of patients with BD is based on the presence of organ involvement and disease severity. Colchicine is a widely used treatment for BD. Corticosteroids and azathioprine can be prescribed if colchicine monotherapy is inadequate. Colchicine is used for the management of mucocutaneous and musculoskeletal findings. Corticosteroids and azathioprine can be com-

Table 1 Diagnostic criteria for Behçet's disease and Crohn's disease

	International Study Group Diagnostic Criteria for Behçet's disease[70]	Proposed diagnostic criteria for Crohn's disease		
		Japan Criteria[72]	Lennard-Jones Criteria[73]	Copenhagen Criteria[8]
Major findings	Recurrent oral ulcerations	A: Longitudinal ulcer B: Cobblestone-like appearance C: Noncaseating epithelioid cell granuloma	Typical diarrhea history for at least 2 mo; 1 Radiological features of CD: segmental distribution, deep ulcerations or cobblestone pattern, thickened bowel wall, coarse mucosal relief, stenotic segments and fistulae; 2 Macroscopic diagnosis by endoscopy: patchy penetrating lesions, fissuring and strictures 3 Fistulas and/or abscesses with typical intestinal disease	1 History of abdominal pain, weight loss and/or diarrhea for more than 3 mo 2 Characteristic endoscopic findings of ulceration (aphtous lesions, snail track ulceration) or cobble stoning or radiological features of stricture or cobble stoning 3 Histopathology consistent with Crohn's disease (epitheloid granuloma of Langerhans type or transmural discontinuous focal or patchy inflammation) 4 Fistula and/or abscess in relation to affected bowel segments
Minor findings	Recurrent genital ulcerations Eye lesions Skin lesions Positive pathergy test	(1) Irregular-shaped and/or quasi-circular ulcers or aphthous ulcerations found extensively in the gastrointestinal tract (2) Characteristic perianal lesions (3) Characteristic gastric and/or duodenal lesions		
Definite	Major finding plus two minor findings	1 Major finding A or B 2 Major finding C, with minor finding (1) or (2) 3 All minor findings (1), (2), and (3)	Positive findings or one positive plus the finding of granuloma	At least two of the criteria present

bined in patients who are unresponsive to colchicine treatment and who have ocular, vascular, neurological, or intestinal involvement. Cyclosporine A and interferon-alpha are immunosuppressive agents used in the management of refractory uveitis and retinal vasculitis. A small number or patients with inadequate response may require mycophenolate mofetil and infliximab. Currently, these agents are used experimentally in the management of vascular involvement. In addition, cyclophosphamide is an effective immunosuppressive agent with increased side effects in patients with arterial, venous and neurological involvement who are refractory to other agents. Other agents that are preferred in unresponsive arthritis with a chronicity tendency include methotrexate and sulfasalazine. The latter is the most widely preferred agent in patients with intestinal BD, after corticosteroids and azathioprine. On the other hand, there are no randomized-controlled clinical trials in BD patients. Observational studies and case series have revealed that steroids, mesalazine, azathioprine, and sulfasalazine are likely to be used in the management of inflammatory bowel diseases. Recently, experience related to the use of anti-TNF agents have increased and some patients respond well to treatment. The efficacy of drugs in the treatment of CD and BD are compared in Table 2. In addition to immunosuppressive agents, antiaggregants, and anti-coagulants can be initiated in patients with venous and neurological involvement. However, no consensus on the use of antiaggregants and anti-coagulants has been reached yet, due to the low embolization tendency of BD-associated thrombosis and high bleeding risk secondary to arterial aneurysms. In clinical practice, these agents are prescribed in patients with low bleeding risk[7,41,76,77].

Corticosteroids have been used in the management of CD for over five decades. Corticosteroids are the most effective therapeutic agents in relieving disease exacerbations. They exert remarkable effects in suppressing pro-inflammatory cytokines and active lymphocytes and inhibiting inflammatory processes of the intestinal lamina propria. Although corticosteroids are more effective in higher concentrations, treatment-related side effects are likely to increase. Prednisolone treatment is usually initiated at 40-60 mg/d and reduced on a gradual basis. Nearly 48%-58% of the patients achieve complete remission, while 26%-32% achieve partial remission following 30 d of treatment. Approximately 16%-20% of patients are unresponsive. Six-mercaptopurine and its pro-drug azathioprine are the most commonly used agents in patients unresponsive to corticosteroids and maintenance therapy. Methotrexate is an alternative agent in patients who are intolerant or unresponsive to these agents. On the other hand, controversial data are available on the efficacy of 5-aminosalicylic acid (5-ASA) preparations. In several meta-analyses, mesalazine 4 g/d significantly reduced disease activity in patients with mild to moderate activity. All these agents are frequently prescribed due to their low side-effect potential[78,79]. Anti-TNF agents including infliximab, adalimumab, and certolizumab pegol can be used in refractory patients with relapsing disease. Meta-analyses have demonstrated that anti-TNF agents are effective as both induction

Table 2 Treatment options for Behçet's and Crohn's disease

	BD		CD	
	Extraintestinal BD	Intestinal BD	Extraintestinal CD	Intestinal CD
Colchicine	S, M, A	-	-	-
Corticosteroids	All manifestations	+	All manifestations	+
Azathioprine	S, M, O, V, N	+	S	+
6-mercaptopurine	-	??	-	+
Cyclosporine A	O	-	-	-
Interferon-alpha	O, N	-	-	-
Mycophenolate Mofetil	O	-	-	-
Cyclophosphamide	O, V, N	-	-	-
Methotrexate	A, N	-	A, S	-
Sulfasalazine	A	+	A	+
Mesalazine	-	+	-	+
Anti-TNF agents	A, O, N	+	A, S, O	+

A: Arthritis; S: Skin; M: Mucosal; O: Ocular; V: Vascular; N: Neurogical Involvement; (+): Effective; (-): Non-Effective; BD: Behçet's Disease; CD: Crohn's disease.

Table 3 Distribution of similarities and differences in the differential diagnosis of Behçet's disease and Crohn's disease[2,3,6,8,9,14,58,60,62,68,81]

	Behçet's Disease	Crohn's Disease
Gender (M/F)	4.9-0.57	2.9-0.76
Symptoms onset age (yr)	20.8-40	15-29
Average age at diagnosis (yr)	24.7-35.7	29.5-31
Oral aphtous ulcers (%)	Approximately 100	< 10
Uveitis (%)	57-69	< 10
Skin lesions (%)	61-87	< 10
Arthritis (%)	30-57	2-24.7
Gastrointestinal involvement (%)		
Ileocecal area	50-94	40-83
Colon	10-15	32-50
Upper GI	1-3	4
Perianal	1-2	10-15
Intestinal complications (%)		
Perforation	12.7	8.7
Fistula	7.6	24.7
Stricture	7.2	38.3
Abscess	3.3	19.6
Endoscopic Morphology	Round-oval shape, Focal, solitary Volcano-shaped Deep ulcers	Longitudinal ulcers with a cobblestone appearance (segmental and diffuse distribution)
Mucosal Biopsy	Vasculitis Neutrophilic infiltration Fibrinopurulent exudates Necrotic debris	Granuloma Focal cryptitis Nerve fiberhyperplasia Lymphoid aggregates

and maintenance therapy in CD patients with fistulizing disease[80]. Surgery is indicated in patients with perianal involvement, fistulas, fissures, and intra-abdominal abscesses.

Medical and surgical management approaches for CD and intestinal BD are similar. Recently, a retrospective case series with long-term outcomes for both diseases was reported[81]. Ten year-follow-up data after diagnosis showed no significant difference in the need for surgery between the study groups with CD and intestinal BD. However, CD patients required a higher dose of corticosteroids and immunosuppressive agents. The doses of biological agents were also higher in CD patients compared to patients with intestinal BD (14.2% *vs* 1.4%). Based on these results, long-term prognosis appears to be similar in patients with CD and intestinal BD.

CONCLUSION

CD primarily involves the gastrointestinal system and can present with various extra-intestinal signs and symptoms. However, BD is a condition or syndrome that presents with multisystem involvement. The gastrointestinal tract is also one of the main sites of involvement in these patients. Both diseases have a true overlap, affecting the gastrointestinal tract. Furthermore, both

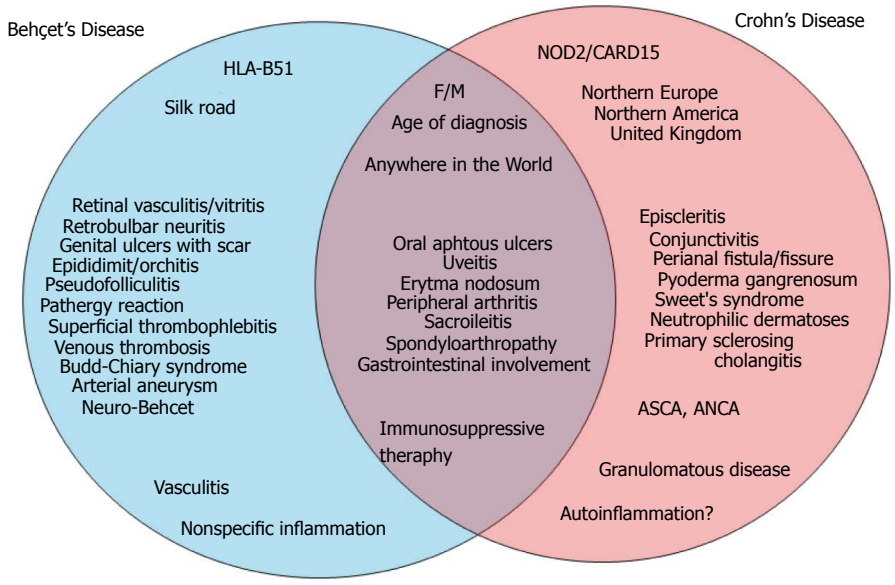

Figure 1 Similar and different characteristics of Behçet's disease and Crohn's disease. F: Female; M: Male; ASCA: Anti-Saccharomyces cerevisiae antibodies; ANCA: Anti-neutrophil cytoplasmic antibodies.

conditions share similar characteristics with respect to age of onset, gender, and inflammation biomarkers such as erythrocyte sedimentation rate and C-reactive protein (increased levels). Despite these similarities, the immunopathogenesis, genetic factors, and regional distribution are quite different. Although both diseases involve similar systems, they have distinct histopathological characteristics. For instance, uveitis is more common in BD, and CD patients are more likely to suffer from episcleritis or conjunctivitis. Figure 1 shows the similarities and differences in BD and CD. Table 3 summarizes the incidence of similarities, the distribution of gastrointestinal involvement, and endoscopic and histopathological differences.

As mentioned above, BD is more common in Asian and Mediterranean populations, while CD is more frequently seen in north European and American individuals. However, given the fact that we live in a globalizing world, the number of patients in whom the differential diagnosis of both conditions is of the utmost importance has increased. Therefore, rheumatologists and gastroenterologists who are mainly involved in the diagnosis and management of BD and CD should be well aware of the typical characteristics of both diseases.

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TOPIC HIGHLIGHT

WJGP 5th Anniversary Special Issues (6): Crohn's disease

Multidisciplinary and evidence-based management of fistulizing perianal Crohn's disease

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Abstract

Perianal symptoms are common in patients with Crohn's disease and cause considerable morbidity. The etiology of these symptoms include skin tags, ulcers, fissures, abscesses, fistulas or stenoses. Fistula is the most common perianal manifestation. Multiple treatment options exist although very few are evidence-based. The phases of treatment include: drainage of infection, assessment of Crohn's disease status and fistula tracts, medical therapy, and selective operative management. The impact of biological therapy on perianal Crohn's disease is uncertain given that outcomes are conflicting. Operative treatment to eradicate the fistula tract can be attempted once infection has resolved and Crohn's disease activity is controlled. The operative approach should be tailored according to the anatomy of the fistula tract. Definitive treatment is challenging with medical and operative treatment rarely leading to true healing with frequent complications and recurrence. Treatment success must be weighed against the risk of complications, specially anal sphincter injury. A full understanding of the etiology and all potential therapeutic options is critical for success. Multidisciplinary management of fistulizing perianal Crohn's disease is crucial to improve outcomes.

Key words: Perianal Crohn's disease; Fistula; Abscess; Management; Review

Core tip: This manuscript is a comprehensive review that focuses on the multidisciplinary management of fistulizing perianal Crohn's disease. The treatment options discussed in this review are based on a current literature review as well as our experience with the disease. Diagnostic and treatment algorithms are also provided.

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INTRODUCTION

Although Gabriel[1] first described patients with granulomatous perianal disease 17 years before the formal description of the disease by Burrill Crohn's[2] in 1932, Bissell[3] was the first to describe the associated perianal manifestations of Crohn's disease (CD). Furthermore, Morson *et al*[4] documented the appearance of perianal non-caseating granulomas and fistulas many years before the onset of intestinal CD.

The reported prevalence of anorectal involvement in patients with CD has varied but the most current population-based series have found involvement in 14 to 38 percent of patients[5-7], with isolated perianal disease seen in only five percent[8]. The prevalence of perianal mani-

festations increases as the disease progresses distally, with up to 92 percent of patients with CD involving the colon and rectum developing fistulas[9]. In most cases, bowel involvement precedes perianal disease[9], but up to 40 percent of patients can experience perianal symptoms before intestinal manifestations[10]. There does not seem to be a predilection for age but a younger age of onset increases the odds of developing perianal disease over time[11-12].

The most common presentation of perianal CD is abscess and fistula. However, patients with CD are frequently affected by other perianal pathologies including hemorrhoids, fissures, skin tags, ulcers, and strictures. Perianal CD has been associated with a disabling natural history[13], with common extraintestinal manifestations[14] and greater steroid resistance[15]. Perianal disease is often recurrent, with 35 to 59 percent of patients relapsing within two years[16]. More than 80 percent of patients require operative treatment, and up to 20 percent may require proctectomy[5,7]. Patients with perianal CD have also shown an increased risk for anal malignancies[17,18], with active and long duration of disease being identified risk factors[19-21].

The treatment of perianal CD continues to be a challenge, especially with the plethora of literature addressing both medical and operative treatment strategies. The purpose of this review is to summarize the efficacy of currently described methods for the management of fistulizing perianal CD and its complications.

ABSCESS

Abscesses usually occur with active perianal CD with an incidence of up to 62 percent during the course of the disease[22]. Ischiorectal abscesses account for 40 percent of all perianal abscesses[23]. Fistula tract location can influence abscess development and transsphincteric fistulas pose the greatest risk[23].

Abscesses are uncommon with superficial fistula tracts. Makowiec *et al*[24] evaluated 61 patients with perianal CD and found that 73 percent of all abscesses were related to an ischiorectal fistula and 50 percent with a transsphincteric fistula. Recurrences occurred in 53 percent with a median time to recurrence of 14 mo. No patients with superficial fistula tracts had a second abscess, whereas about two thirds of patients with transsphincteric and ischiorectal fistulas recurred after 36 mo.

A detailed anorectal exam should be performed before any type of treatment is initiated. This frequently requires evaluation under anesthesia (EUA) with evaluation of the rectum to rule-out active disease. Perianal infection can occur in any anatomic plane (superficial, intersphincteric, ischiorectal, or supralevator), and requires immediate drainage and treatment of systemic symptoms with broad-spectrum antibiotics[6,25]. Many authors recommend drain placement or partial sphincter division to facilitate drainage, but these have not been associated with better outcomes[26,27]. In the setting of persistent perianal sepsis, imaging modalities such as magnetic resonance imaging (MRI) and computed tomography (CT) are used to guide the drainage of deep or complex abscesses[28,29].

When a fistula is encountered, a non-cutting seton should be placed to facilitate drainage and prevent recurrent infection, with improvement seen in 79 to 100 percent of patients[30-35]. Long-term drainage with non-cutting setons without definitive therapy has been reported to result in fistula recurrence in 20 to 80 percent of cases[33,36,37]. The combination of non-cutting setons and anti-tumor necrosis factor (TNF) therapy has been associated with fistula healing rates of up to 67 percent and will be discussed below[38,39]. Fecal diversion to increase fistula healing and control perianal sepsis continues to be controversial with no level A data supporting its role but in the setting of persistent perianal sepsis, a temporary diverting stoma can be effective. Patients should be aware that these stomas are rarely reversed[40].

Cryptoglandular abscess/fistulas can and do occur in patients with CD and should be recognized as so because treatment differs. These abscess/fistulas tend to be superficial and are not associated with active anorectal CD; therefore, anti-TNF therapy is not indicated. Abscess drainage should follow the same principles as mentioned above. Placement of a non-cutting seton is encouraged and any attempt of local surgical treatment should take into consideration the patients underlying continence and CD status. Supplemental imaging studies, such as endoanal ultrasound (EAUS), are very helpful even when cryptoglandular etiology is suspected.

FISTULA

A population-based study[7] with up to 20 years of follow-up showed that one out every two patients with CD develop perianal fistulas. The etiology of perianal fistula formation in CD is not completely clear but genetic, microbiological, and immunological factors play a role. Most authors believe that fistulas originate either from the penetration of a rectal ulcer or from cryptitis spreading to the intersphincteric space. Intersphincteric and transsphincteric fistulas are the most common fistula tracts of cryptoglandular origin that occur in patients with CD. Suprasphincteric fistulas result from cryptoglandular disease or rectal ulceration, and extra sphincteric fistulas are frequently seen in patients with severe proctitis or iatrogenic injury.

At St. Marks Hospital, Tozer[41] studied biopsy samples from Crohn's and idiopathic anal fistulas. Although immunological analysis showed no significant differences in interleukin (IL)-2, IL-4, IL-6, IL-10, TNF, and interferon levels, CD patients had significantly higher interleukin 17 levels and significantly lower CD65 levels. The authors showed data suggesting aberrant expression of homing molecules on dendritic cells in Crohn's anal fistulas suggesting a non-directed immune response, which may contribute to the pathophysiology.

Pelvic MRI is the preferred imaging study to assess fistulizing perianal disease. It has an accuracy of 90 percent for evaluating fistula tracts and of 97 percent for characterizing complex abscesses[42,43]. Furthermore, operative management may be altered in ten to 20 percent of patients by the addition of MRI to EUA, and this increases to up to 40 percent in patients with CD[44,45].

Once the anorectal disease is delineated, evaluation for proximal CD with endoscopy should also be considered. Although some studies have found an association between proximal fistulizing disease and perianal fistulas[46], other investigators have not observed this finding[47,48]. In patients with fistulizing perianal CD it is our practice to combine a pelvic MRI with EUA and rigid proctoscopy to evaluate for rectal inflammation.

Medical treatment

Once perianal infection is controlled, the fistula tract is characterized, and CD status is assessed; combined definitive medical and surgical therapy should be initiated (Figure 1). When active proctitis is encountered, this must be aggressively treated. Medical therapy includes antibiotics (metronidozole and ciprofloxacin), immunosuppressives (6-mercaptopurine, azathioprine, cyclosporine, and tacrolimus), and immunomodulators (infliximab, adalimumab, and certolizumab pegol). Although steroids are frequently used to manage concomitant luminal disease, there is no demonstrable role for corticosteroids in perianal CD. Medical treatment of perianal CD demands significant cooperation between gastroenterologists and surgeons as patient management is challenging and requires frequent feedback between medical professionals to optimize therapeutic strategies.

Antibiotic therapy

Antibiotics are commonly initiated when perianal infection is diagnosed and are frequently continued until immunosuppressive therapy is initiated[49], with 70 to 95 percent of patients having a positive response within six weeks[50,51]. It is our practice to continue antibiotic therapy for two weeks with perianal infection, and for three to four weeks with active proctitis. Metronidazole is the most common antibiotic prescribed for perianal CD and has been associated with fistula healing rates ranging from zero to 56 percent[50,52,53]. Seventy-five percent of patients relapse after suspending treatment and side effects which include nausea and peripheral neuropathy commonly limit its long-term use.

Ciprofloxacin has been studied in small, uncontrolled series of patients with perianal CD[54,55]. Improvement has been shown in approximately half of patients without detailed data on fistula healing. Ciprofloxacin was compared to metronidazole and placebo in a small randomized study including 25 patients[53]. After receiving treatment for ten weeks, clinical remission and response were 30 percent and 40 percent with ciprofloxacin, 12.5 percent and 12.5 percent with placebo, and 0 percent and 14 percent with metronidazole; none of these differences being significant.

Immunomodulators

The definitive medical treatment of perianal CD includes immunomodulation. A meta-analysis of five randomized controlled trials evaluated the efficacy of 6-mercaptopurine and azathioprine[56]. Fistula healing occurred in 54 percent of patients *vs* 21 percent of controls (OR 3.09; 95%CI, 2.45 to 3.91). Intravenous cyclosporine has also shown to have a good response in up to 83 percent of patients[57,58], but the effect is short-lasting when it is discontinued or transitioned to oral formulations[59]. Tacrolimus has also been effective in the treatment of perianal CD, as shown in one randomized controlled trial. Clinical improvement was seen in 43 percent of patients *vs* 8 percent receiving placebo ($P = 0.004$)[60].

Anti-TNF therapy

Anti-TNF therapy, which includes monoclonal antibodies that are given intravenously [Infliximab (chimeric – murine/human)] or subcutaneously [Adalimumab and Certolizumab pegol (human)], has shown good results in the multidisciplinary management of perianal CD. Most patients who receive anti-TNF therapy receive concomitant immunomodulators. This combination has been poorly studied, specifically in perianal CD, but may be associated with less perianal complications and increased fistula healing[61]. What must be taken into consideration is that most studies evaluating anti-TNF therapy in the setting of perianal CD are of small numbers that involve heterogeneous patient groups with short follow-up. These studies also use varying definitions of fistula healing, disease improvement and "response".

Infliximab alone: Present *et al*[62] reported that three infusions of infliximab resulted in closure of perianal fistulas in 46 percent of patients over 3 mo follow-up. A large study from Hungary including 148 patients with CD reported a perianal fistula closure rate of 49 percent at a mean of 3 mo follow-up[63]. A multicenter Italian study evaluating the impact of infliximab alone in 188 patients with perianal CD reported an initial response in 76 percent of patients with a 44 percent fistula closure rate[64]. Ng *et al*[65] prospectively evaluated the response to infliximab therapy with MRI in 34 patients with perianal Crohn's fistulas. At six months, 58 percent of patients showed fistula closure, with 37 percent showing good clinical response.

Infliximab plus surgery: Regueiro *et al*[66] demonstrated an improved clinical response and less fistula recurrence when patients had EUA and seton placement before starting infliximab compared to patients who received infliximab alone. Topstad *et al*[38] also achieved improved outcomes with combined seton drainage, infliximab infusion, and immunosuppressives in 29 patients. At a mean follow-up of nine months, 67 percent of patients showed a complete response. Hyder *et al*[67] evaluated

long-term healing rates with this approach in 22 patients. At a median follow-up of 21 mo, the authors only observed an 18% fistula closure rate. Van der Hagen *et al*[68] developed a multistep multidisciplinary approach that involved EUA with seton placement, fecal diversion when fistulas and abscesses recurred, infliximab therapy in case of persistent proctitis, and definitive fistula surgery. At a mean follow-up of 23 mo, fistula healing occurred in 90 percent of patients who received infliximab (9/10) compared to 71 percent in those who did not (5/7).

At the University of Minnesota, Gaertner *et al*[39] evaluated the outcomes of 226 patients who underwent operative treatment for perianal Crohn's fistulas, with 79 of these patients also receiving preoperative infliximab. Fistula healing rates were similar regardless of infliximab therapy (59% *vs* 60%). However, patients who underwent surgery plus infliximab healed faster than those who did not receive infliximab (6.5 mo *vs* 12.1 mo; $P < 0.0001$), and seton placement plus infliximab infusion resulted in significantly improved fistula healing rates compared to seton placement alone ($P = 0.001$). Regardless of infliximab therapy, lay-open fistulotomy was the operation with the best healing rates. Active proctitis did not significantly impact healing after fistula surgery.

Adalimumab alone: Adalimumab has shown similar efficacy to infliximab in randomized controlled trials. In the CHARM (Crohn's trial of the fully Human Antibody Adalimumab for Remission Maintenance) study, 113 patients with perianal Crohn's fistulas received induction adalimumab; with subsequent maintenance adalimumab or placebo[69]. Evaluation at 26 wk showed complete fistula closure in 30 percent of patients treated with adalimumab, with improved outcomes at 56 wk compared to placebo (33% *vs* 13%). The durability of these results have been confirmed at two years follow-up[70]. In the CLASSIC-1 (Clinical Assessment of Adalimumab Safety and Efficacy Studied as an Induction Therapy in Crohn's disease) trial, adalimumab was compared to placebo with the aim to evaluate short-term outcomes[71]. Thirty-two of 299 patients had perianal fistulas and no significant differences were observed in fistula healing.

Adalimumab has also been used in patients who have failed to respond to other anti-TNF agents, specially infliximab. In the GAIN (Gauging Adalimumab efficacy in Infliximab Nonresponders) trial, CD patients who were intolerant or who had lost response to infliximab received adalimumab or placebo[72]. Forty-five of 325 patients had perianal fistulas and no significant differences in fistula healing were found between placebo and adalimumab. Based on these results, most physicians consider that a second biological agent has minimal efficacy in patients who have already failed anti-TNF therapy.

Adalimumab plus surgery: As the experience with anti-TNF therapy expands, many authors have reported on a combined approach with adalimumab and local anorectal procedures. Tozer *et al*[73] reviewed the outcomes of 41 consecutive patients with fistulizing perianal CD treated with infliximab ($n = 32$) or adalimumab ($n = 9$), and followed radiologically with MRI. Fifty-eight percent of all patients (66% infliximab and 43% adalimumab) demonstrated remission or response at three years. Fistula healing, as demonstrated by MRI, lagged behind clinical healing by a median of 12 mo. All patients who achieved radiological healing maintained fistula closure while on anti-TNF therapy but only 43 percent maintained fistula closure after cessation of anti-TNF agents. El-Gazzaz *et al*[74] reviewed the Cleveland Clinic experience with combined anti-TNF therapy and anorectal surgery in 218 patients. Mean follow-up was 3.2 years. Two hundred and eighteen patients underwent operative treatment, 101 with anti-TNF therapy (74 infliximab and 27 adalimumab). Patient groups were comparable in demographic data and CD history but operative treatment was significantly heterogeneous. Patients who received combined anti-TNF therapy and surgery had significant overall improvement compared to patients who underwent surgery alone (36% *vs* 71%, $P = 0.001$).

Local anti-TNF therapy: Local injections of anti-TNF agents have also been attempted in fistulizing perianal CD, specifically in patients with contraindications to systemic treatment and resistance to infliximab. Poggioli *et al*[75] performed three to 12 local injections of infliximab (15-20 mg) adjacent to both internal and external openings and fistulous tract in 15 patients. Fistula closure occurred in ten patients at a mean follow-up of 18 mo. Asteria *et al*[76] achieved clinical response in six of eleven patients treated with local infliximab. Four of the eleven remained healed at a median of ten months of follow-up.

Tonelli *et al*[77] reviewed the outcomes of 12 patients with fistulizing perianal CD who underwent local injection of Adalimumab. Each patient received a median of seven (range, 4-16) injections. At a mean follow-up of 17.5 mo, 75 percent of patients (9 of 12) no longer had fistula drainage, and three patients (25%) showed clinical improvement. No adverse side effects were noted.

Certolizumab pegol: Certolizumab pegol is a humanized monoclonal antibody directed against TNF alpha. The antibody is fused with polyethylene glycol, which does not cross the placenta, so it should be safe in pregnancy. In 2008, the Food and Drug Administration approved Certolizumab pegol for the treatment of CD. Schreiber *et al*[78] evaluated its impact in patients with fistulizing CD. Patients with fistulizing CD from a randomized controlled study (PRECiSE 2, $n = 108$) comparing certolizumab pegol *vs* placebo were further randomized (if a good initial response was noted) to certolizumab pegol ($n = 28$) or placebo ($n = 30$) every four weeks until 24 wk. The majority of patients (55/58) had perianal fistulas. At week 26, fistula closure occurred in 36 percent of patients in the certolizumab pegol group compared to 17 percent of patients receiving placebo ($P = 0.038$).

Operative treatment

If the attempt to heal a fistula has significant impact on

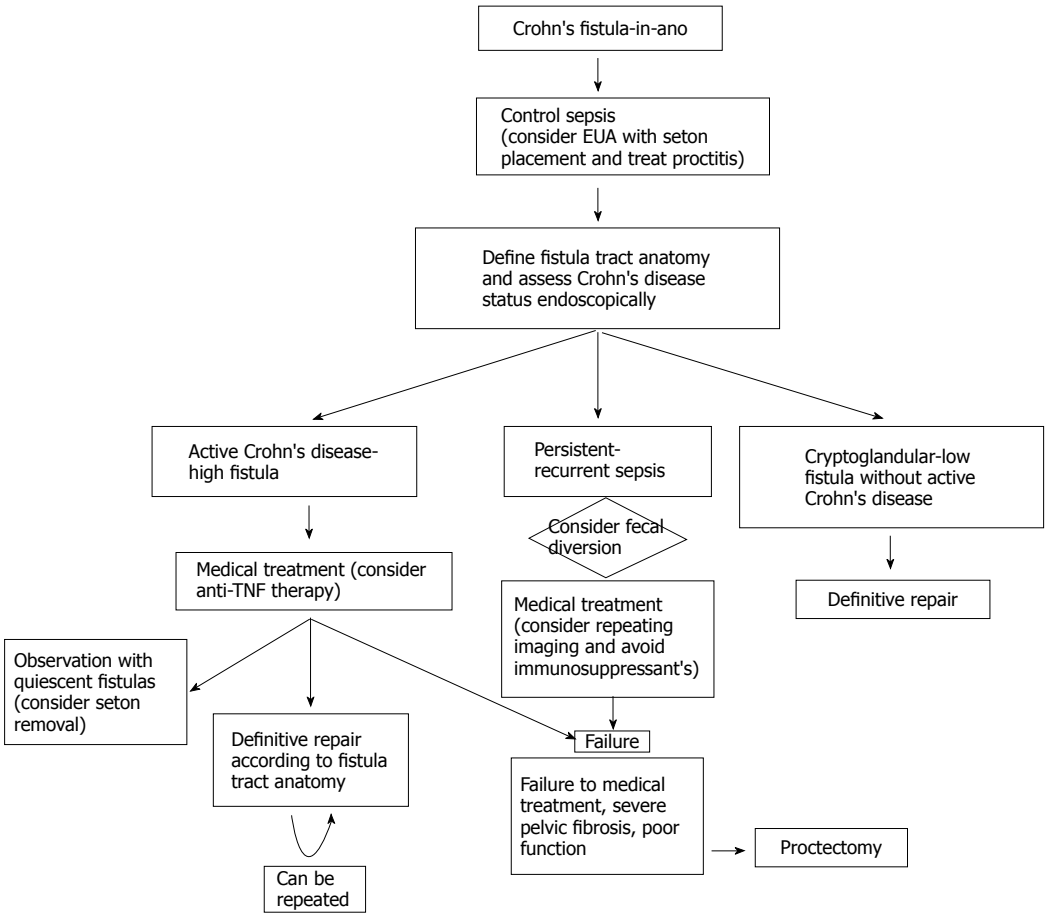

Figure 1 Diagnostic and treatment algorithm for fistulizing perianal Crohn's disease.

a patient's quality of life, operative treatment should be undertaken. Currently, the majority of operations for fistulizing perianal CD are performed in conjunction with medical therapy (immunomodulators or anti-TNF agents), and because this approach has been covered above, this section will focus on operative indications and efficacy of the most popular surgical techniques.

Most low, simple fistulas can be treated by fistulotomy. Healing rates from 80 to 100 percent have been reported with this technique[27,31,79,80]. Despite careful patient selection, an occasional fistulotomy wound may result in a chronic ulcer. In this situation, medical treatment is preferred as further operations have been associated with recurrent infection, fistula, and sphincter damage.

If partial sphincter division would compromise fecal continence, one can choose between minimally invasive techniques and anorectal repairs. Minimally invasive techniques include fibrin glue injection and collagen plug insertion. These techniques have no significant effect on fecal continence, are well tolerated by the patient, can be repeated, and are associated with fistula healing rates between 38 and 71 percent[81-84]. Fistula recurrence is common and occurs in approximately 50 to 70 percent of patients[81-84]. Video-assisted anal fistula treatment (VAAFT) and local injection of adipose-derived stem cells are recently described minimally invasive techniques that have been employed in patients with fistulizing perianal CD[85,86]. VAAFT involves performing fistuloscopy to identify the etiologic crypt and rule-out secondary tracts and then excise the internal opening. After this, the fistula tract is fulgurated. Stem cell therapy is a novel and promising approach for the treatment of chronic inflammatory conditions, and its use in fistulizing perianal CD has increased in Europe. Fistula healing rates between 30 and 82 percent have been reported with these techniques but the long-term safety and outcomes have not been adequately studied in the Crohn's population. Overall, studies assessing the efficacy of minimally invasive techniques for Crohn's perianal fistulas tend to be of small patient numbers, non-comparative and heterogeneous patient groups, retrospective nature, and with short duration of follow-up.

The most commonly employed anorectal operation for transsphincteric Crohn's fistulas is a rectal advancement flap. This procedure has been associated with incontinence rates between five and nine percent but has not been associated with an increased risk for proctectomy[87]. Contraindications include significant proctitis, a cavitating ulcer or anal stenosis. Crohn's fistula healing rates reported in the literature average 64 percent[87]. A recently described technique, ligation of intersphinc-

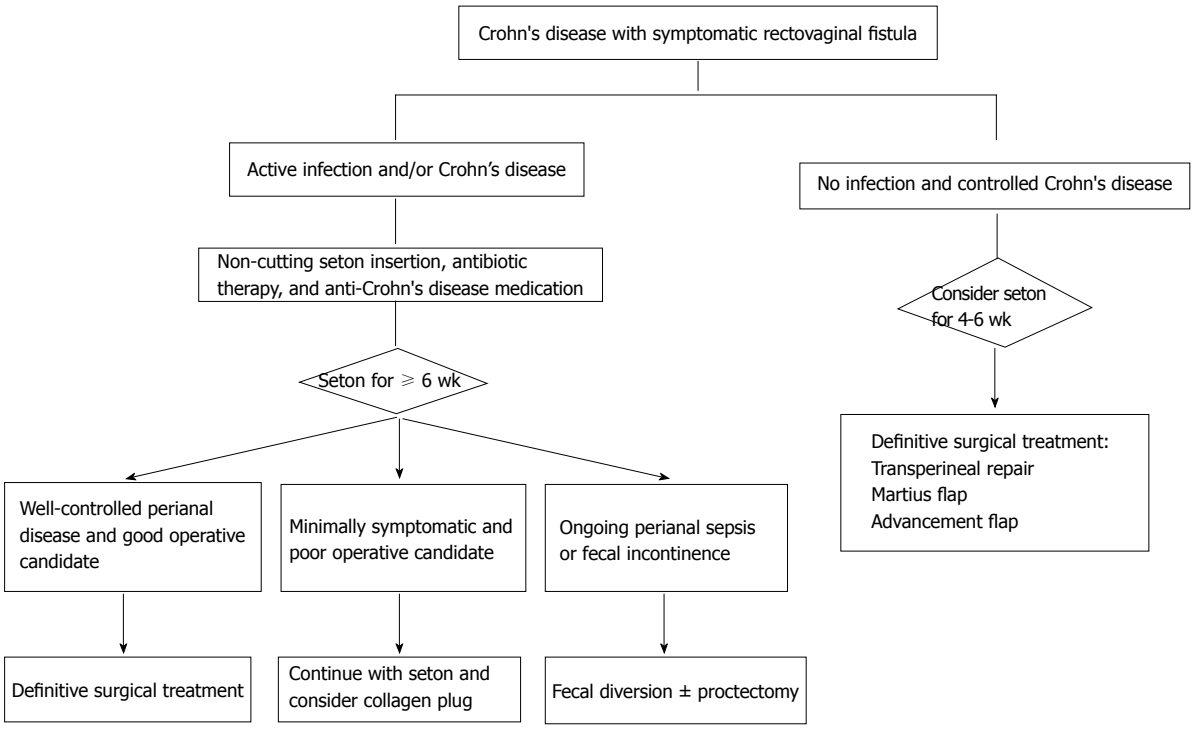

Figure 2 Treatment algorithm for patients with Crohn's disease and symptomatic recto-vaginal fistulae.

teric fistula tract (LIFT) which involves the identification and ligation/transection of the fistula tract in the intersphincteric plane, is being increasingly employed in patients with transsphincteric Crohn's fistulas[88]. This technique also has minimal to no repercussion on fecal continence but does involve a perianal wound. Although encouraging results have been reported in complex fistulas of cryptoglandular origin, experience in CD patients is limited[89,90].

In the setting of a large anal canal ulcer or severe stricture, an endorectal advancement flap can be performed in selective patients[91]. After the ulcer or stricture is excised, a full-thickness circumferential sleeve is mobilized and a formal rectoanal anastomosis is performed in combination with a diverting loop ileostomy.

SPECIFIC SITUATIONS

Rectovaginal fistulas

After obstetric trauma, CD is the second most common cause of rectovaginal fistula (RVF)[92], occurring in five to 23 percent of CD patients[93-95]. The majority of RVF's in the setting of CD are low and transsphincteric, and arise from rectal ulceration or infection of anterior anal glands[94,96].

The management of RVF in CD is challenging. Treatment depends on the degree of symptoms, CD activity, and the anatomy of the fistulous tract (Figure 2). Minimally symptomatic patients may not require any treatment[7,94,97,98]. However, carefully selected symptomatic patients should be treated with a step-wise multidisciplinary approach. Drainage of local infection, seton placement and medical therapy are the initial steps before any attempts at fistula closure[92,94].

Patient selection is very important. Women with extensive anorectal CD are not good candidates for definitive fistula operations without first eradicating local infection and controlling the activity of underlying CD. Contrast to what has been reported in non-CD patients, a previous failed repair does not dictate a worse outcome with a subsequent operation. Healing rates reported after secondary operations are similar to those seen after a first attempt repair (29%-54%)[99-101]. Fecal diversion to protect a repair is also controversial. Penninckx *et al*[99] evaluated the impact of fecal diversion and parenteral nutrition in 32 consecutive patients undergoing RVF repair and did not find any significant role for either of these interventions. However, when O'Leary *et al*[102] selectively used fecal diversion in a step-wise approach that included initial seton placement and delayed repair, fistula healing occurred in 80 percent of patients. A diverting stoma does not ensure fistula healing and should only be performed in complex and recurrent cases.

Most of current treatment algorithms include combined medical and operative treatment. Present *et al*[103] found that 6-mercaptopurine was more effective than placebo, when combined with surgery (31% *vs* 6%). Most RVF's recurred after discontinuation of 6-mercaptopurine. Similar results were observed with cyclosporine in two studies that included a total of six patients with RVF[104,105].

El-Gazzaz *et al*[106] evaluated long-term outcomes in 65 women with Crohn's RVF´s who underwent a variety of different procedures. At a median follow-up of

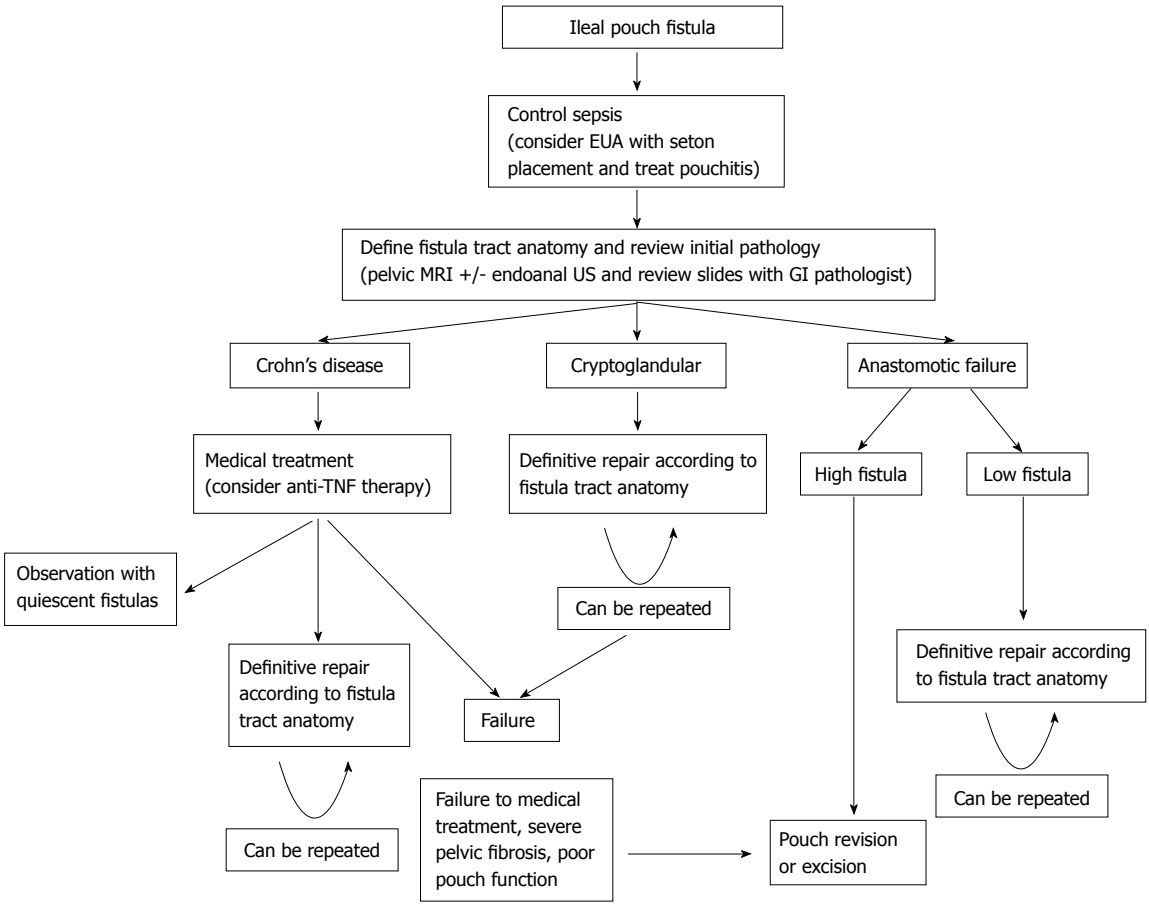

Figure 3 Diagnostic and treatment algorithm for patients with ileal pouch fistulas.

47 mo, 46 percent healed. Multivariate analysis showed that immunomodulators were associated with successful healing ($P = 0.009$); and smoking and steroids were associated with failure ($P = 0.04$).

The efficacy of infliximab in RVF and CD has been controversial[38,62,66-68,107-109]. In the ACCENT Ⅱ study[109], the initial response rate to infliximab was 64 percent. Rectovaginal fistula closure was maintained for longer with maintenance infliximab compared to placebo (46 wk *vs* 33 wk). Gaertner *et al*[110] reviewed the outcomes of 51 patients with Crohn's RVF's who underwent combined medical and operative treatment, 26 received preoperative infliximab. At a mean follow up of 38.6 mo, 27 fistulas (53%) healed. Transperineal repair was the operation with the highest healing rate regardless of infliximab therapy. Preoperative fecal diversion, active proctitis and infliximab therapy did not significantly impact fistula healing.

The definition of fistula healing tends to raise controversy when reviewing the RVF literature and seems to be influenced by the type of treatment, method of evaluation, and follow-up period. Rasul *et al*[111] assessed RVF healing by endoanal ultrasound in patients who clinically healed with infliximab therapy. Only five of 35 women demonstrated improvement but none showed fistula closure on ultrasound. Bell *et al*[112] found good correlation between clinical assessment and MRI in seven of ten patients treated with infliximab. Only two of these patients had RVF.

Ileal pouch fistulas

Patients who develop CD after restorative proctocolectomy with ileoanal anastomosis are at particularly high risk of developing pouch-anal fistulas. Although preoperative colorectal pathology, operative technique, and postoperative pelvic sepsis have also been identified as risk factors, CD is considered the most common[113-115]. Several operative techniques have been described to control pelvic and perianal sepsis and ultimately eliminate the fistula tract[116-120], but because of the low incidence of these fistulas, the optimal management continues to be controversial (Figure 3). Gaertner *et al*[121] reviewed the outcomes of 25 patients who presented with symptomatic ileal pouch fistulas over a 22-year period. Fistulas were classified as pouch-anal (48%), pouch-vaginal (28%), complex (16%), and pouch-perineal (8%). The most common etiology was CD. Overall fistula closure with a variety of local anorectal and abdominal procedures was 64 percent at a median follow-up of 29 mo. Postoperative pelvic sepsis, fecal diversion, and anti-TNF therapy did not significantly impact fistula healing. Three patients (12%) required pouch excision with end ileostomy.

Fistula-associated cancer

In 1934, Rosser[122] first described carcinoma associated

with a chronic perianal fistula. Fistula-associated adenocarcinoma is a rare but increasingly reported malignancy[18,21,123-131] that is commonly found in CD patients with chronic anal fistulas[18,21]. This malignancy is frequently associated with chronic, complex fistulas and can be particularly difficult to diagnose. High clinical suspicion is crucial to avoid any delay in diagnosis and treatment. Chronic infection and inflammation (*i.e.*, CD and radiation) are the most frequently associated risk factors but even when the diagnosis is suspected clinically, confirmation requires EUA with biopsy. Misdiagnosis commonly occurs in elderly patients and patients with long-standing anorectal disease. Once the diagnosis of cancer has been established, EAUS and MRI are recommended for staging[132].

Mucinous adenocarcinoma is the most common malignancy reported in long-standing perianal fistulas. It is typically a slow growing, locally aggressive neoplasm that mainly spreads *via* the inguinal lymphatic's[133]. Outcomes are good when malignancy is diagnosed early[131,133-136]. Oncologic resection remains the standard treatment option. Abdominoperineal resection is the most frequently employed operation[131,137,138]. The role of neoadjuvant chemoradiotherapy in the treatment of this neoplasm has not been well studied, probably because of its rarity, but results are promising[21,131]. Neoadjuvant therapy may play a significant role to improve outcomes but remains investigational.

Gaertner *et al*[131] identified 14 patients with fistula-associated anal adenocarcinoma. The most common presentation was persistent perianal fistula (n = 9). Ten patients (71%) had CD. Abdominoperineal resection was performed in eleven patients, seven following neoadjuvant chemoradiotherapy. At a mean follow-up of 64 mo, ten patients were alive without evidence of disease and four patients died with metastatic disease. All seven patients who received neoadjuvant chemoradiotherapy had a complete pathologic response. In a systematic review by Iesalnieks *et al*[21], a total of 23 publications including 65 patients with fistula-associated adenocarcinoma and CD were reviewed. Abdominoperineal resection was performed in 56 patients with an overall 3-year survival rate of 54 percent.

We recommend that tissue from refractory, recurrent and chronic anal fistula tracts, regardless of their etiology, should be submitted for pathologic evaluation. All patients with long-standing perianal CD should undergo cancer surveillance. Although the impact of neoadjuvant chemoradiotherapy remains controversial, oncologic resection continues to be the standard treatment option for fistula-associated adenocarcinoma.

Proctectomy

Proctectomy is appropriate in patients in whom repeated medical and operative strategies fail. Historically, it is required in ten to 20 percent of patients with perianal CD[6], and is commonly associated with perineal wound breakdown, chronic open wounds and sinus formation in up to half of patients[139,140]. In our experience, intersphincteric proctectomy (when feasible) and the use of rectus abdominal and gracilis flaps can help with avoiding these complications.

A low Hartmann's procedure is an alternative approach that may result in a healed perineum in up to 60 percent of patients with perianal CD[141]. Despite this approach, Guillem *et al*[142] reported a 54 percent completion proctectomy rate in 28 patients who underwent rectal exclusion, plus an additional nine patients had persistent active disease at the rectal stump.

CONCLUSION

The appropriate treatment of fistulizing perianal CD must be individualized to each patient. The primary goals are to ameliorate symptoms and prevent complications. Overall, a less aggressive approach is preferred as many patients will require repetitive operations that can often result in outcomes that are worse than the disease itself.

Based on the current literature, multidisciplinary treatment includes: eradication of infection, assessment of CD status and fistula tract(s), medical therapy, and selective operative management. The first phase of treatment is to drain the perianal infection. This typically involves an EUA, seton drainage and a short course of antibiotics. The second phase consists of endoscopically evaluating the extent of CD and delimiting the anatomy of the fistula tract with EUA and either EAUS or MRI, or both. During this phase, medical therapy with immunomodulators and anti-TNF agents is typically initiated but if the fistula is thought to be of cryptoglandular etiology, CD medications are rarely required.

The third phase should ideally involve healing of the perianal pathology. Many patients who have minimal symptoms elect to continue with a non-cutting seton or removal and expect healing in some cases. On many occasions a non-cutting seton may actually act as a cutting seton, specially in low superficial fistula tracts. The extensive range of operations highlights the complexity of operative treatment. These include a variety of minimally invasive techniques and anorectal operations. Sphincter injury and fecal incontinence should be the main concern with any anorectal operation. The operative approach depends on the anatomy of the fistula tract, CD status, and the patients' functional status. Attempts to heal a fistula in the setting of active infection and proctitis are likely to fail. If the patient's symptoms persist or increase despite adequate medical and surgical treatment, a diverting stoma or proctectomy should be considered.

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REVIEW

Pancreatitis-imaging approach

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Abstract

Pancreatitis is defined as the inflammation of the pancreas and considered the most common pancreatic disease in children and adults. Imaging plays a significant role in the diagnosis, severity assessment, recognition of complications and guiding therapeutic interventions. In the setting of pancreatitis, wider availability and good image quality make multi-detector contrast-enhanced computed tomography (MD-CECT) the most used imaging technique. However, magnetic resonance imaging (MRI) offers diagnostic capabilities similar to those of CT, with additional intrinsic advantages including lack of ionizing radiation and exquisite soft tissue characterization. This article reviews the proposed definitions of revised Atlanta classification for acute pancreatitis, illustrates a wide range of morphologic pancreatic parenchymal and associated peripancreatic changes for different types of acute pancreatitis. It also describes the spectrum of early and late chronic pancreatitis imaging findings and illustrates some of the less common types of chronic pancreatitis, with special emphasis on the role of CT and MRI.

Key words: Computed tomography; Magnetic resonance imaging; Acute pancreatitis; Chronic pancreatitis; Autoimmune pancreatitis; Chronic pancreatitis; Revised Atlanta classification; Motion-resistant imaging

Core tip: Imaging plays an important role in the diagnosis and staging of acute and chronic pancreatitis. Wider availability and good image quality makes computed tomography (CT) the mostly used imaging technique; however, magnetic resonance imaging (MRI) offers diagnostic capabilities similar to those of CT, with additional intrinsic advantages including lack of ionizing radiation and exquisite soft tissue characterization. This article reviews and illustrates the proposed definitions of the revised Atlanta classification for acute pancreatitis. It also describes the spectrum of early and late chronic pancreatitis imaging findings, with special emphasis on the role of CT and MRI.

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INTRODUCTION

Pancreatitis is defined as the inflammation of the pancreas and considered the most common pancreatic disease in children and adults. It can be acute; representing an acute inflammatory process of the pancreas, or chronic; progressing slowly with continued, permanent inflammatory injury to the pancreas.

The incidence of acute pancreatitis is increasing in the United States and worldwide contributing to be one of the major sources of hospitalization. Acute pancreatitis was the most common gastrointestinal diagnosis for hospitalization (with 274119 discharges) in the United

States in 2009[1], usually running a mild clinical course[2]. However, a subset of patients develop severe disease independent of the degree of initial insult or etiology, with high morbidity and mortality up to 45%[3]. Over one-half of cases of acute pancreatitis in adults are related to cholelithiasis or alcohol consumption; whereas trauma, viral infections and systemic diseases account for the majority of cases in children[4].

The incidence of chronic pancreatitis is between five and twelve cases per 100000 persons per year; accounting for more than 120000 outpatient visits and 50000 hospitalizations annually[5]. Alcohol consumption accounts for the majority (80%) of cases of chronic pancreatitis in adults in developed countries; whereas malnutrition is the most common cause worldwide[4].

The purpose of our review is to illustrate the different imaging findings of pancreatitis on computed tomography (CT) and magnetic resonance imaging (MRI); with special emphasis on the revised terminology for acute pancreatitis and substantiate the increasing importance of imaging in the diagnosis, staging and follow-up of acute and chronic pancreatitis[5].

Acute pancreatitis

Acute pancreatitis results from the exudation of fluid containing activated proteolytic enzymes into the interstitium of the pancreas and leakage of this fluid into surrounding tissue.

There is general acceptance that a diagnosis of acute pancreatitis requires two of the following three features: (1) Sudden onset abdominal pain suggestive of acute pancreatitis (epigastric pain radiating to the back); (2) Serum amylase and/or lipase levels at least 3 times greater than the upper limit of normal; and (3) Characteristic imaging findings of acute pancreatitis on contrast-enhanced computerized tomography (CECT), MRI, or transabdominal ultrasonography (US) studies.

If abdominal pain is strongly suggestive of acute pancreatitis but the serum amylase and/or lipase activity is less than 3 times the upper limit of normal, characteristic findings on a CECT or MRI are required to confirm the diagnosis[6].

In order to assess and predict local or systemic effects of pancreatic injury, several disease severity-scoring systems were developed (*e.g.*, Ranson score, APACHE-II). In 1992, the Atlanta classification for acute pancreatitis was introduced to establish international standards of definitions of acute pancreatitis and its complications[7]. This system was designed to facilitate understanding and correlation of findings seen by gastroenterologists, pathologists, radiologists and surgeons; aiding improved communication between clinicians.

This initial Atlanta classification system represented major progress; however, advancing knowledge of the disease process, improved imaging and ever-changing treatment options warranted a revision, which was undertaken in 2012.

The revision of the Atlanta classification focuses heavily on morphologic criteria for defining the various manifestations of acute pancreatitis as outlined principally by means of CT and MRI.

Two distinct phases of acute pancreatitis were introduced: a first, or early, phase that occurs within the 1st wk of onset of disease; and a second, or late, phase that takes place after the 1st week of onset[7].

Early or first phase (less than 1 wk)

During this phase, pancreatic or peripancreatic ischemia or edema may completely resolve, develop fluid collections or progress to permanent necrosis and liquefaction. Severity of the acute pancreatitis in the early phase is entirely based on clinical parameters; mainly determined by the presence and duration of organ failure, but not the morphologic characteristics and its extent in and around the pancreas[8].

Late or second phase (after 1 wk from onset)

This phase occurs mostly in patients with moderate to severe acute pancreatitis and may extend for weeks to months. It is characterized by the presence of local complications, systemic manifestations (due to ongoing inflammation) and/or by transient or persistent organ failure. In this stage, the need for treatment is determined by presence of symptoms or complications, and the type of management is mainly based on the morphologic characteristics of pancreatic and peripancreatic region seen on cross sectional imaging. The severity of acute pancreatitis in late phase is determined by both morphologic criteria and clinical criteria like persistence of organ failure.

Updated terminology of acute pancreatitis

The web based international consensus[7] revised the original Atlanta classification of 1992 and proposed a new classification of acute pancreatitis to avoid the confusion in terminology seen over the last 2 decades. This consensus classification defines criteria for the diagnosis of acute pancreatitis (see above), differentiates the two types of acute pancreatitis (interstitial edematous pancreatitis and necrotizing pancreatitis) classifies the severity of acute pancreatitis into three categories and defines the morphology seen on imaging of pancreatic and peripancreatic collections that arise as complications of acute pancreatitis.

Role of imaging in acute pancreatitis

Imaging plays a significant role in the diagnosis of acute pancreatitis in clinically suspected cases or suggesting alternative diagnoses. It helps determine the causes of pancreatitis: gallstones, biliary duct obstruction or structural abnormalities. It also helps in grading the severity of the disease and identifying pancreatic or peripancreatic complications. Additionally, imaging can be utilized to guide therapeutic interventions.

The choice of appropriate imaging modality depends on the reason for investigation, clinical symptoms,

Table 1 Indications to perform contrast-enhanced computed tomography[58]

Types	Indications
Initial imaging	1 When the diagnosis of acute pancreatitis is uncertain
	2 Patients with hyperamylasemia, severe clinical pancreatitis, abdominal distention and tenderness, fever > 102°, and leukocytosis for the detection of complications
	3 Ranson score > 3 or APACHE score > 8
	4 Patients who fail to improve after 72 h of conservative medical therapy
	5 Acute change in clinical status, such as new fever, pain, and shock after successful initial medical therapy
Followup imaging	1 Acute change in clinical status suggesting complication
	2 7-10 d after presentation if CT severity score is 3-10 at presentation or grade
	3 To determine response to treatment after surgery or interventional radiologic procedures to document response to treatment.
	4 Before discharge of patients with severe acute pancreatitis

time of onset of symptoms and lab findings. However, CECT is the most commonly used modality in the evaluation of acute pancreatitis. In 2010, the ACR committee on appropriateness criteria and its expert panels have developed guidelines for determining the most appropriate imaging examinations for the diagnosis and treatment of acute pancreatitis and have given high score ratings[8,9] to CECT in different clinical scenarios. This is based on it is wide availability and high degree of accuracy. They also stated that MRI appears to offer diagnostic capabilities similar to multi-detector computed tomography (MDCT) with intrinsic advantages including the lack of ionizing radiation and the exquisite soft tissue characterization unmatched by any other imaging modality; allowing better depiction of stones and evaluation of the pancreaticobiliary ductal system.

Ultrasound

Ultrasound is frequently the first investigation performed on admission; although it has little value in the diagnosis of pancreatitis or its complications. Ultrasound is usually reserved to confirm or exclude the presence of stones or biliary dilatation. Early identification and treatment of these calculi may have a significant positive impact on outcome. However, body habitus of patient, operator dependence pose a limitation in detection of distal common bile duct stones accurately compared to CECT or MR imaging[9].

Ultrasound is limited in evaluating the entire pancreatic parenchyma; which is often partially or completely obscured by overlying bowel gas. It can however be helpful in monitoring the evolution of fluid collections, which occur as a result of acute pancreatitis, and in guiding diagnostic and therapeutic interventions.

CECT

CECT can show morphologic characteristic findings that allow for establishing the diagnosis of acute pancreatitis and determining the extent of disease severity. The best time for performing CECT in acute pancreatitis not well established and if performed immediately after the onset of symptoms, the full extent of pancreatic damage and its severity can be easily underestimated[10,11]. Conversely, a CECT obtained more than 5 d after onset of symptoms that reveals a normal aspect of the pancreas or only mild inflammatory changes (fat stranding) surrounding the pancreas virtually excludes a severe form of acute pancreatitis[12].

Not all patients with acute pancreatitis need to undergo contrast-enhanced CT. In general, CT is not indicated in patients who are clinically classified as having mild pancreatitis (no clinical signs of severe pancreatitis) and show rapid improvement with appropriate medical management. CT should be used in patients who are classified as having severe pancreatitis or are at risk of developing severe pancreatitis; ideally after 72 h, to best assess the full extent of the disease[13].

CT should be repeated when the clinical picture drastically changes, such as with sudden onset of fever, decrease in hematocrit or sepsis. CT can also be useful to guide catheter placement for drainage and to assess success of treatment in patients who underwent percutaneous drainage or other interventions.

Furthermore, in patients with their first episode of pancreatitis who are over 40 years of age and have no identifiable cause for pancreatitis, contrast-enhanced CT should be used to exclude a possible neoplasm[13] (Table 1).

The main limiting factors for CECT are ionizing radiation, use of iodinated contrast material; especially in patients in with renal failure or contrast allergy and moderate sensitivity in identifying gallstones and biliary stones[14,15]. The above limiting factors can be overcome by using MRI; which does not use ionizing radiation; allowing it to be used during pregnancy, in patients with recurrent pancreatitis and for patients requiring multiple follow-up exams. Non-enhanced MRI seems to be more accurate and reliable for the early assessment of severity and prognosis of acute pancreatitis than is contrast-enhanced CT[16-18]; thus proving beneficial in patients with renal failure and history of contrast allergy.

MRI

Recent technological developments have dramatically improved the quality of abdominal MRI. Respiration, bowel peristalsis and vascular pulsations are major sources for artifacts affecting the accuracy and reproducibility of MRI. Breathing-independent sequences and respira-

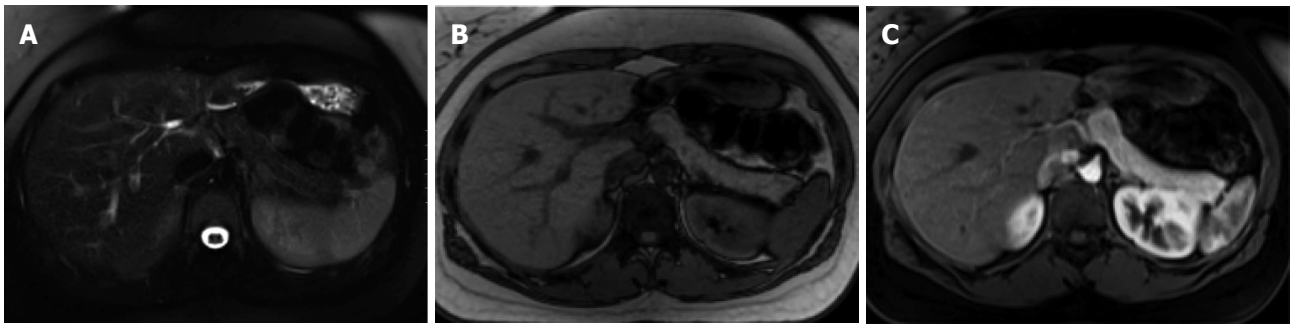

Figure 1 Normal pancreatic appearance on magnetic resonance imaging. A: Axial T2-weighted image with fat-suppression; B: Axial GRE out-of-phase T1-weighted image; C: Axial post-contrast 3D-GRE T1-weighted image with fat-suppression during the late arterial phase. The pancreas demonstrates low T2 signal intensity (A) and high T1 signal intensity on pre-contrast images (B), reflecting high protein content of the exocrine gland. The pancreas demonstrates avid homogenous enhancement on immediate post-contrast images (C), reflecting a normal capillary blush.

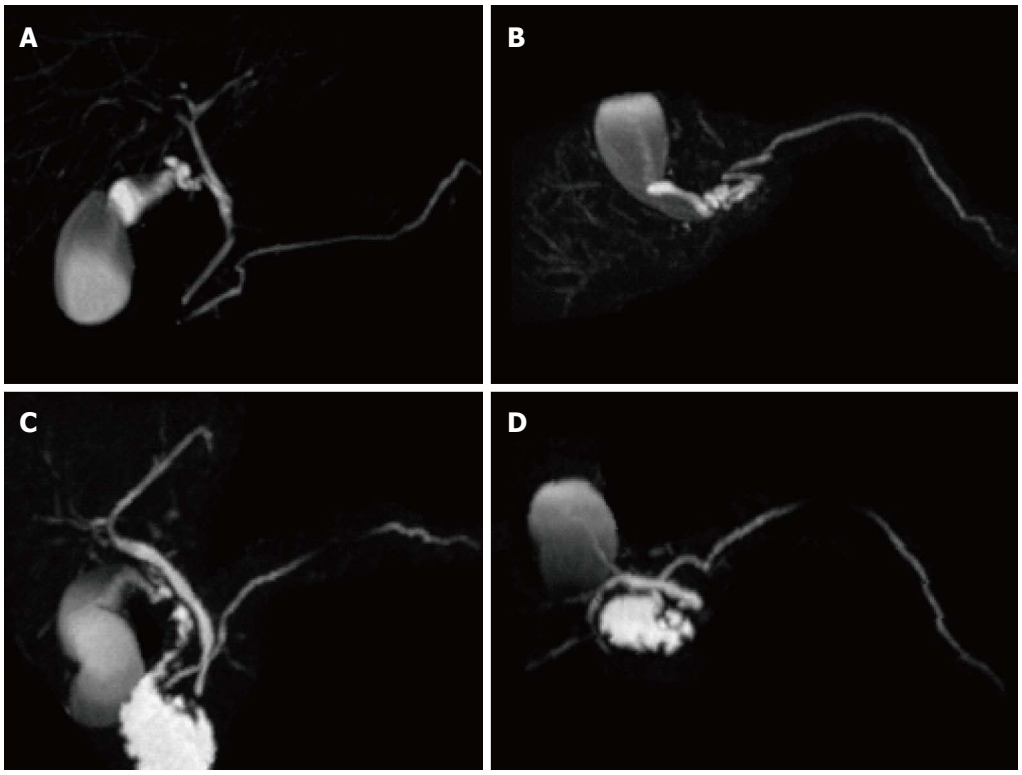

Figure 2 Normal pancreatic duct anatomy and pancreatic divisum. (A and C) Coronal and (B and D) axial post-processed maximum intensity projection 3D-MRCP images from two different patients. In the first patient, the main pancreatic duct courses inferiorly (A) and posteriorly (B), joins the CBD and opens in the major papilla in keeping with normal pancreatic duct anatomy. In the second patient, the main pancreatic duct continues its course superiorly (C) and anteriorly (D), crosses the CBD and opens in the minor papilla in keeping with pancreatic divisum.

tory gating techniques form the foundation of high-quality abdominopelvic MRI. New motion-robust MRI techniques provide promising results even in detection and characterization of pancreatic disease in patients that are not able to cooperate with breath-hold instructions[19].

A variety of pulse sequences are currently used for abdominal MRI including T1- and T2-weighted sequences with or without fat-suppression and post-gadolinium T1-weighted sequences (Figure 1). MR Cholangiopancreatography (MRCP) is routinely added to abdominal protocols to assess ductal obstruction, dilatation or course[20-22] (Figure 2); providing comprehensive evaluation of full range of pancreatic diseases. Due to the increasing incidence of acute pancreatitis due to gallstones in the United States, it is more beneficial to consider MRCP as an initial diagnostic study.

MRI is sensitive for detection of subtle changes of acute pancreatitis; particularly minor peripancreatic inflammatory changes; even in the setting of a morphologically normal pancreas on CT imaging; which may appear normal in up to 15%-30% of patients with clinical features of acute pancreatitis[23]. The sensitivity of MRI exceeds that of CT imaging, emphasizing its role in

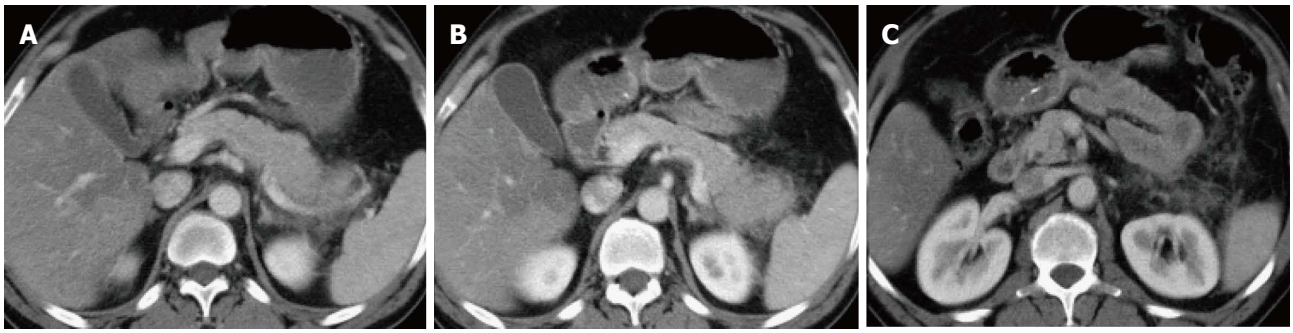

Figure 3 Focal acute edematous pancreatic tail pancreatitis. A-C: Axial CT scan of the pancreas during the late arterial phase. There is evidence of ill-definition and reduced enhancement of the pancreatic tail (A and B), associated with mild peripancreatic fatty stranding extending to the anterior left perinephric space in keeping with focal acute edematous pancreatitis.

the evaluation of patients with clinically suspected acute pancreatitis and negative CT imaging findings.

It should be emphasized that MRI is a non-ionizing cross sectional imaging method and has a safer intravenous contrast profile in comparison to CT. This is particularly important in radiosensitive populations and those requiring repeated imaging follow up. Additionally, patients who present with acute pancreatitis often have a degree of renal impairment.

The factors that make CECT the most frequently applied imaging approach in pancreatitis are related to its universal availability (especially near the emergency room), faster scanning times, and relatively easier interpretability of CT images by physicians and general radiologists. For early presentation of acute pancreatitis, CT might be the preferred method for the reasons stated above. However, the adequate diagnostic performance of MRI along with the mentioned additive advantages favors MRI as the preferred method.

Endoscopic ultrasound

Endoscopic ultrasound (EUS) has shown great utility in providing high-resolution images of the pancreatic duct and parenchyma as well as extra hepatic biliary system; as the probe can be positioned in close proximity to the pancreas. Furthermore, EUS has become an invaluable technique for its ability to obtain targeted biopsies of lesions in and around the pancreas; thus, playing a prominent role in evaluating patients with atypical findings on other imaging studies.

The disadvantages of EUS are the requirement of monitored anesthesia care, need for expert endo-sonographer, modality operator dependence, and interobserver variability.

According to ACR appropriateness criteria, the role of endoscopic US in the evaluation of acute pancreatitis is primarily reserved for assessing and/or confirming choledocolithiasis and subsequent stone removal, as well as for identifying anatomic abnormalities (*e.g.*, pancreas divisum or malignancy) that can lead to acute pancreatitis. However, it has been recently proposed to use EUS in acute pancreatitis, as it is was found to contribute for the detection of causes like cancer, microlithiasis and chronic pancreatitis[24].

IMAGING-BASED MORPHOLOGIC TYPES OF ACUTE PANCREATITIS

Interstitial edematous pancreatitis

Interstitial edematous pancreatitis (IEP) is a milder form of acute pancreatitis that usually resolves over the first week. IEP is characterized by diffuse or localized enlargement of the pancreas secondary to interstitial or inflammatory edema without necrosis.

On CECT, findings include enlarged pancreas with relatively normal enhancement. Peripancreatic fat may be normal or show mild stranding and ground glass opacity due to inflammation, with small to varying amounts of non-enhancing peripancreatic fluid (Figure 3). The characteristic CECT finding that distinguishes IEP is absence of pancreatic parenchymal and peripancreatic necrosis.

On MRI, the signal intensity characteristics of the pancreas in IEP resemble those of normal pancreatic tissue. Enlargement of the pancreas, parenchymal edema and fat stranding are well demonstrated on T1-weighted images (Figure 4). T1-weighted imaging with fat suppression improves the delineation of the pancreas and pancreatic borders[25]. The pancreas demonstrates high signal intensity on pre-contrast fat suppressed T1-weighted images and enhances uniformly on immediate post-gadolinium images, reflecting a normal capillary blush. Fat suppressed T2-weighted sequences are very sensitive for detecting edema or minimal fluid and therefore have a role in detecting even milder forms of pancreatitis[26](Figure 5).

Necrotizing pancreatitis

Necrotizing pancreatitis is the inflammation of the pancreas with obvious pancreatic and peripancreatic tissue necrosis. About 5%-10% of patients develop necrosis; affecting the pancreatic parenchyma in 5%, peripancreatic tissue in 20% and both in 70%. Pancreatic parenchymal necrosis carries a worse prognosis than peripancreatic necrosis[27].

Atlanta classification defines necrotizing pancreatitis as being associated with more than 30% parenchymal necrosis. The presence of less than 30% necrosis demands

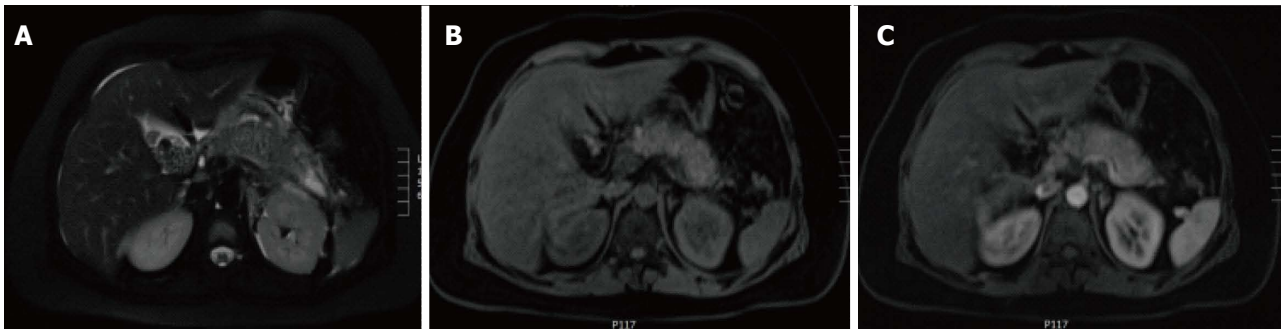

Figure 4 Gallstone acute edematous pancreatic tail pancreatitis. A: Axial fast spin-echo (FSE) T2-weighted image with fat-suppression; B and C: Post-contrast 3D-GRE T1-weighted images with fat-suppression during the late arterial and portal venous phases. There is mild diffuse lace-like increased T2 signal involving the pancreatic parenchyma, associated with a small amount of peripancreatic fluid near the pancreatic tail (A). The pancreas demonstrates diffuse minimal decrease in T1 signal intensity (B) and minimally reduced enhancement on the late arterial phase (C) in keeping with diffuse edematous pancreatitis. There are also innumerable gallstones (A).

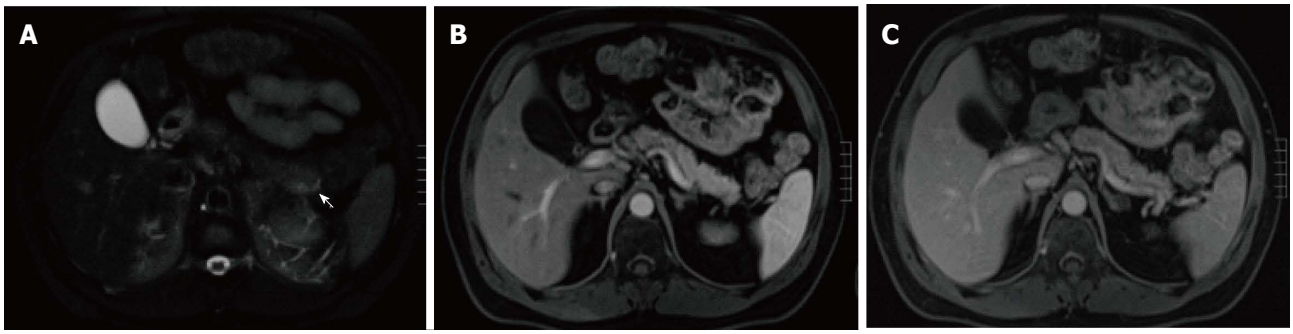

Figure 5 Subtle focal acute edematous pancreatic tail pancreatitis. A: Axial T2 weighted-image with fat-suppression; B and C: Axial post-contrast 3D-GRE T1-weighted images with fat-suppression during the late arterial and portal venous phases. There is a very subtle area of increased T2 signal seen around the pancreatic tail (arrow, A), with fairly normal enhancement of the pancreas on the post-contrast images (B and C) in keeping with subtle focal acute edematous pancreatic tail pancreatitis.

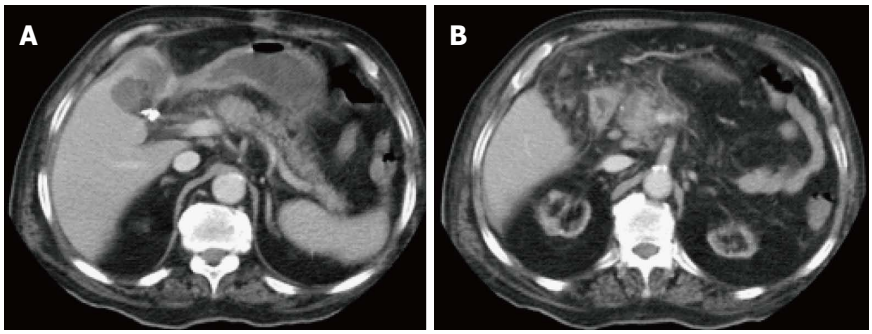

Figure 6 Focal pancreatic head necrotizing pancreatitis confined to the pancreatic parenchyma. A and B: Axial CT scan during the portal venous phase. There is evidence of significantly reduced enhancement of the pancreatic head (B), without peripancreatic extension or necrosis in keeping with focal acute necrotizing pancreatitis.

follow-up scanning in 1 wk to confirm true necrosis *vs* IEP[27].

On CECT, findings include areas of compromised pancreatic parenchymal enhancement on the post-Gadolinium images with or without peripancreatic inhomogeneous fluid collections (Figures 6 and 7). The impairment of pancreatic perfusion and signs of peripancreatic necrosis evolve over several days[28], which explains why an early CECT may underestimate the eventual extent of pancreatic and peripancreatic necrosis.

On MRI, necrosis shows appears as hypointense areas on T1-weighted images corresponding to areas of increased signal on fat-suppressed T2 weighted-images, associated with well defined areas of non-enhancing pancreatic parenchyma on post-Gadolinium sequences[29-31] (Figure 8).

For both CT and MRI, acquisition of an adequate arterial phase is of the utmost importance; as the maximum enhancement of pancreas is reached on the late arterial phase, and higher difference in signal between

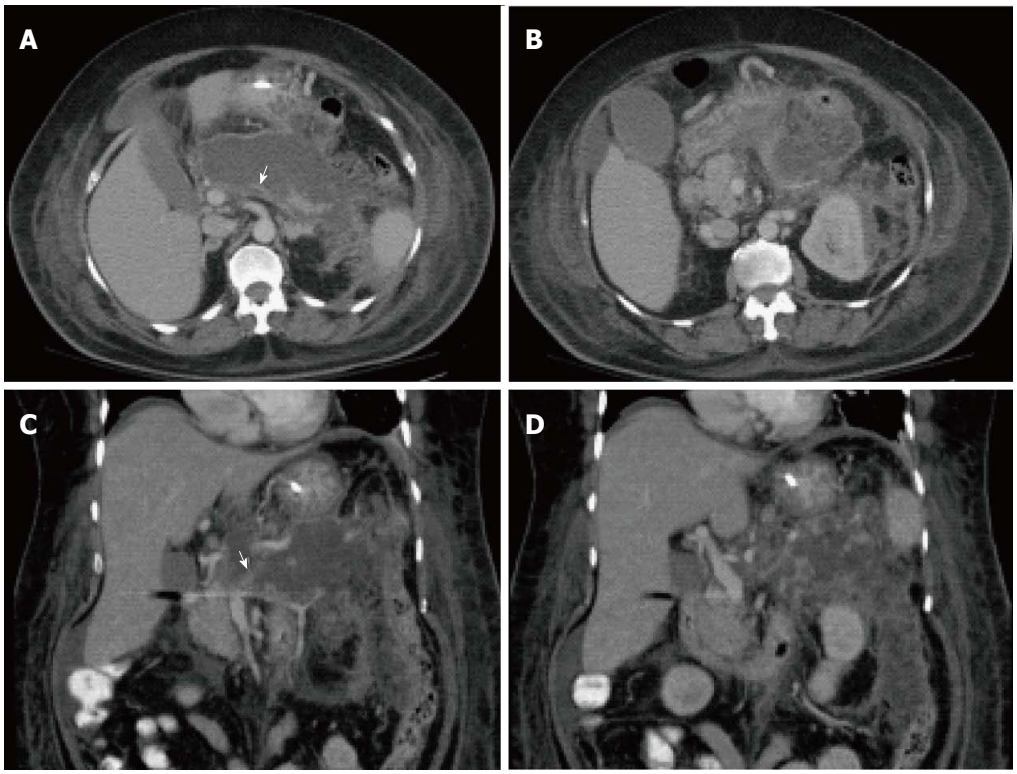

Figure 7 Severe acute necrotizing pancreatitis and peri pancreatitis. A-B: Axial CT scan during the late arterial phase; C-D: Coronal reformatted CT images. There is evidence of lack of arterial enhancement involving the pancreatic body and tail, which are replaced by necrotic tissue, associated with heterogenous peripancreatic tissue inflammation and necrosis extending to left perinephric space (A-B) and paracolic gutter (C-D), in keeping with severe necrotizing pancreatitis and peripancreatitis. There is also evidence of splenic vein thrombosis (arrow, A, C), a known complication of acute pancreatitis.

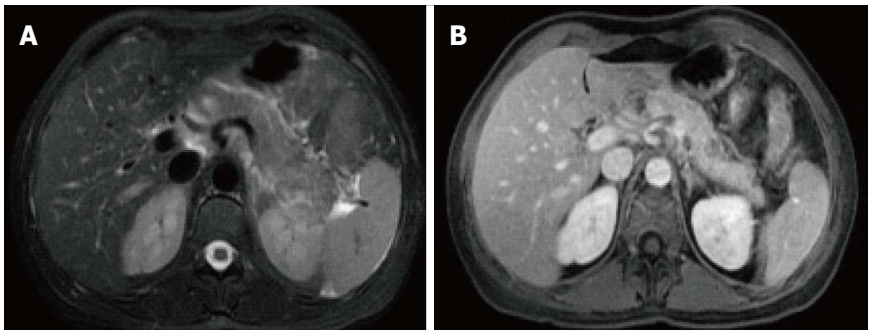

Figure 8 Focal acute necrotizing pancreatitis. A: Axial fast spin-echo T2- weighted image with fat-suppression; B: Axial post-contrast 3D-GRE T1- weighted images with fat-suppression during the venous phase. There is a focal area of low T2 signal involving the proximal part of the pancreatic tail, associated with minimal peripancreatic fat stranding (A). This focal area demonstrates significantly reduced enhancement on the post-contrast images, in keeping with focal necrotizing pancreatitis.

viable and necrotic is achieved on this phase.

Pancreatic duct disruption is an important prognostic factor. It is seen in 30% of the patients of necrotizing pancreatitis[32] when necrosis involves the central gland[33,34]. Drake *et al*[35] study showed that MRCP, a noninvasive imaging method, achieved 95% accuracy in detecting pancreatic duct disruption; thus helping in identifying patients who might benefit from early treatment.

Definition of pancreatic and peripancreatic collections

An important distinction is made between collections that are composed of fluid alone and those that arise from necrosis and contain a solid component (and which may also contain varying amounts of fluid). Below, we define and illustrate the following terms: acute peripancreatic fluid collection; occurring in interstitial edematous pancreatitis, pancreatic pseudocyst as a delayed (usually after 4 wk) complication of interstitial edematous pancreatitis and necrosis; which may be an acute necrotic collection (in the early phase and before demarcation) or walled-off necrosis surrounded by an identifiable capsule on imaging (rarely develops before 4 wk).

Acute peripancreatic fluid collections

Fluid collections less than 4 wk in IEP lacking a discrete

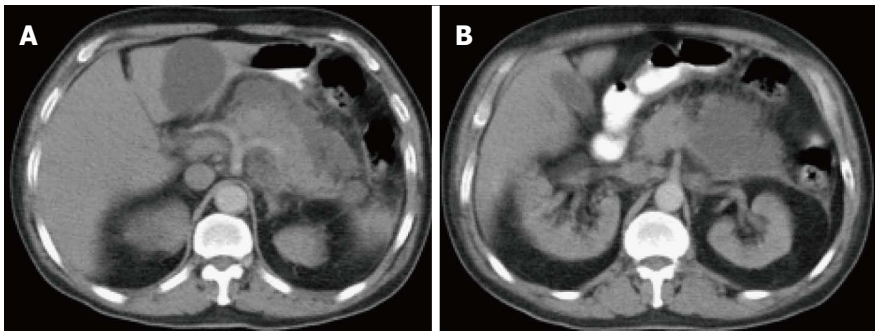

Figure 9 Acute interstitial edematous pancreatitis and acute peripancreatic fluid collections. A-B: Axial CT scan during the portal venous phase. The pancreas is mildly thickened and demonstrates mildly heterogenous enhancement, reflective of edema, in keeping with acute interstitial edematous pancreatitis. There is a peripancreatic fluid with imperceptible wall in keeping with acute peripancreatic fluid collections.

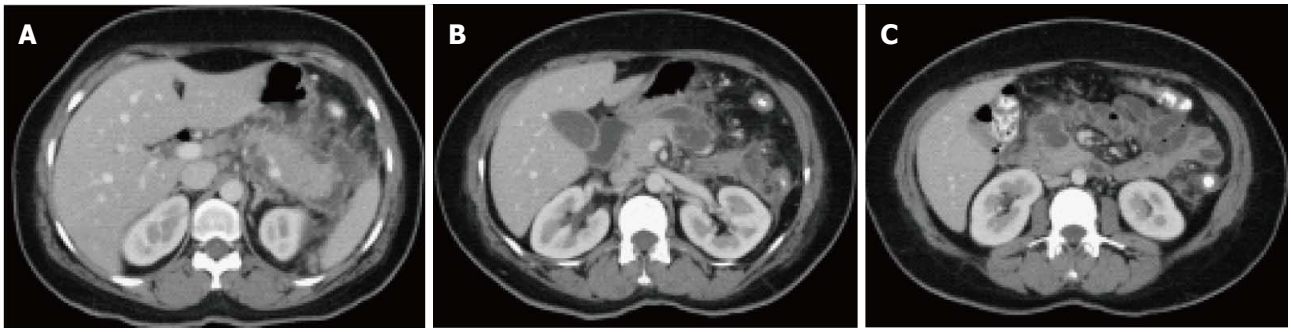

Figure 10 Peripancreatic fluid secondary to multifocal acute necrotizing pancreatitis. A-C: Axial CT images during the late arterial phase. There are two areas of focal necrosis involving the pancreatic body (B) and pancreatic head/uncinate process (C), associated with loculated peripancreatic fluid collection (A).

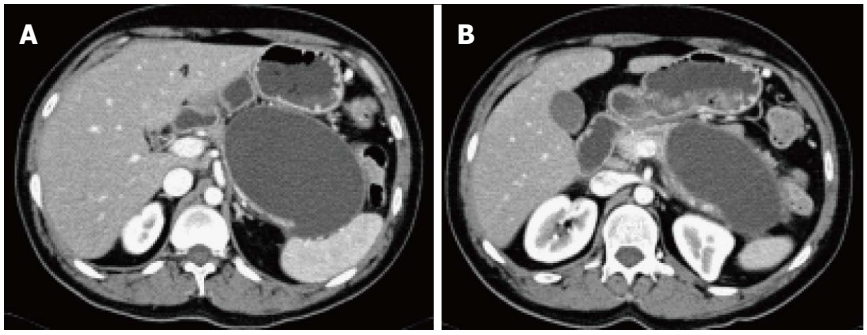

Figure 11 Large pancreatic pseudocyst. A-B: Axial CT scan during the late arterial phase. There is a large oval shaped pancreatic pseudocyst located anterior to the pancreatic body and tail, associated with mass effect on the thinned out pancreatic tissue in keeping with a large pancreatic pseudocyst.

wall, with no internal solid components in the peripancreatic region are called acute peripancreatic fluid collections (APFC). Approximately 50% of APFC's develop within 48 h following the onset of acute pancreatitis[36].

On CT scan, they appear as homogenous collections with low attenuation. They do not have well-defined walls and are confined by normal fascial planes in the retroperitoneum (Figure 9). They can be single or multiple (Figure 10). Most acute fluid collections remain sterile and usually resolve spontaneously without intervention[30].

On MRI, T2-weighted sequences are very sensitive in detecting peripancreatic fluid; which demonstrate high T2 signal intensity. On T1-weighted gradient echo images, APFC's demonstrate low signal intensity in a background of high signal intensity fat. No perceptible enhancement is depicted on post-gadolinium fat-suppressed T1-weighted images. The majority of fluid collections are typically confined to the lesser sac and anterior pararenal space or may track down to the pelvis and superiorly into mediastinum[29]. These collections are usually sterile and are spontaneously reabsorbed.

Pancreatic pseudocysts

Peripancreatic fluid collections that persist more than 4 wk in IEP, with a well-defined wall and no internal solid components in the peripancreatic region are called pseudocysts.

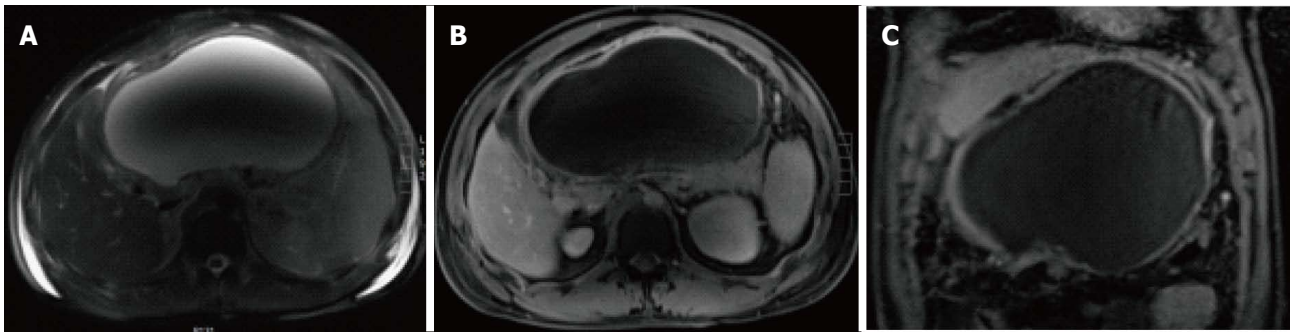

Figure 12 Large pancreatic pseudocyst. A: Axial fast spin-echo T2- weighted image with fat-suppression; B-C: Axial and coronal post-contrast 3D- GRE T1-weighted images with fat-suppression during the portal venous phase. There is a very large thin-walled cyst (A) within the lesser sac; which demonstrates mild uniform wall enhancement (B-C) in keeping with a large pancreatic pseudocyst. The central drop of signal on (A) is related to dielectric shading artifact.

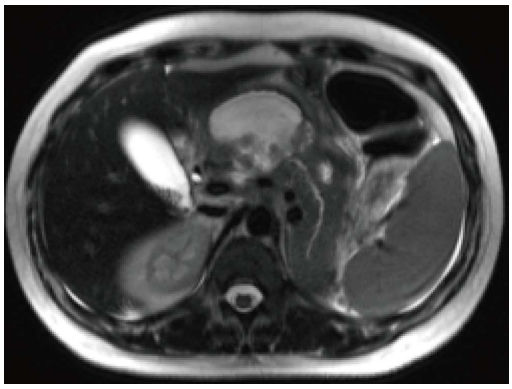

Figure 13 Acute necrotic collection. Axial T2 single-shot turbo spin-echo image. There is a well-defined fluid collection involving the pancreatic neck with peripancreatic extension and communication with the main pancreatic duct. This collection demonstrates well-defined outlines and heterogenous low T2 signal intensity debris within it in keeping with acute necrotic collection. Multiple gallstones are also noted.

On CECT, they appear as homogenous collections of low-attenuation surrounded by a uniform enhancing capsule (Figure 11). Typically, an increase of enhancement is observed in the interstitial phase; reflecting the presence of granulation tissue.

On MRI, pseudocysts demonstrate low in signal intensity on and T1-weighted gradient-echo images and relatively homogeneous high signal intensity on T2-weighted images. Pseudocysts walls enhance minimally on early post-gadolinium images and show progressively intense enhancement on 5-min post-gadolinium images; due to the presence of fibrous tissue (Figure 12).

Pseudocysts may sometime have communication with pancreatic duct and detecting this communication is helpful in the further patients' management. MRCP; a noninvasive imaging modality has an advantage of demonstrating possible communication between pancreatic pseudocyst and pancreatic duct.

The majority of pseudocysts resolve spontaneously. Infection and hemorrhage may complicate simple pseudocysts. Infected pseudocyst may contain gas bubbles on CT. However, absence of these findings on CT may further require confirmation by fine needle aspiration, when there is a strong clinical suspicion.

Acute necrotic collections

During the first 4 wk, a collection containing variable amounts of fluid and necrotic tissue is termed an acute necrotic collection (ANC). Unlike APFCs, ANCs are present within the pancreas and peripancreatic regions. ANC's may often maintain communication with the main pancreatic duct or one of its side-branches; for which, MRI can be useful in delineating this connection.

On CECT, ANC's demonstrate heterogeneous attenuation variably higher that of thin fluid (Figure 7). Follow-up imaging may be useful to characterize acute collections. CECT often shows ANC's as a homogenous non-enhancing area during the first week of necrotizing pancreatitis; making it difficult to be differentiated from APFC's. MRI may be helpful to confirm the presence of solid content in the collection.

On MRI, the necrotic debris may appear as irregularly shaped regions of low signal intensity within the necrotic collections. Breathing-independent T2-weighted sequences such as single-shot echo-train spin echo are useful to evaluate these necrotic collections (Figure 13); not only because of their high sensitivity in demonstrating the complexity of fluid, but also because many of these patients are very debilitated and are unable to co-operate with breath-holding instructions.

An advantage of MRI relative to MDCT in the evaluation of peripancreatic fluid collections is easier appreciation of solid debris with MRI[37]. The sensitivity and specificity of MRI in detecting solid debris of necrosis is 100% when compared to CT; which has a sensitivity of 25% and a specificity of 100%[38]. MRI can help in differentiating fluid collections secondary to pancreatitis from other cystic neoplasms.

Walled-off necrosis

After 4 wk, APFC's mature and develop thick non-epithelialized wall; acquiring the term walled-off necrosis (WON). They commonly occur in the pancreatic body and tail. Management for WON is different from pseudocyst as it contains non-liquefied debris; which needs to be surgically removed. Previously suggested nomenclature for this entity includes: organized pancreatic necrosis, pancreatic sequestration, pseudocyst associated

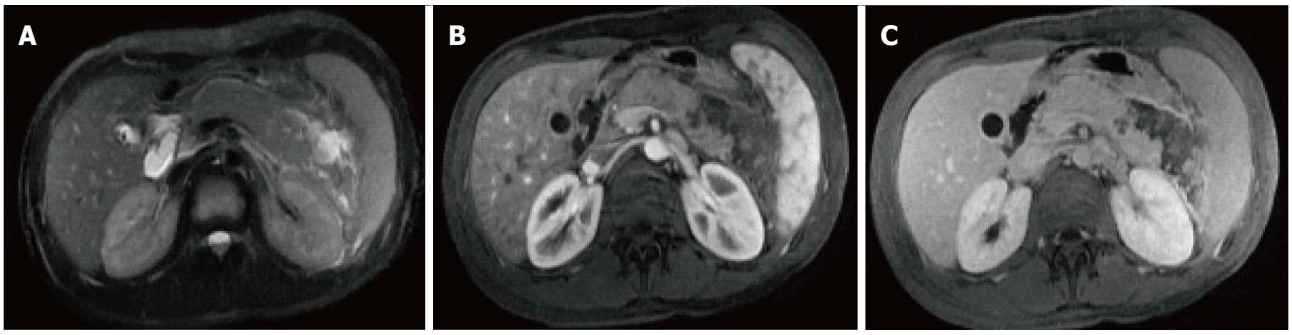

Figure 14 Necrotizing pancreatitis, with peripancreatic walled-off necrosis. A: Axial fast spin-echo T2-weighted image with fat-suppression; B-C: Axial post-contrast 3D-GRE T1-weighted images with fat-suppression during the late arterial and venous phase. There is a focal area of heterogeneous iso to slightly high T2 signal involving the pancreatic body-tail junction (A); which demonstrates lack of enhancement on the post-contrast images (B-C). There is associated sizable peripancreatic fluid collection; which demonstrates heterogeneous T2 signal intensity and thick enhancing wall post-contrast in keeping with walled-off necrosis.

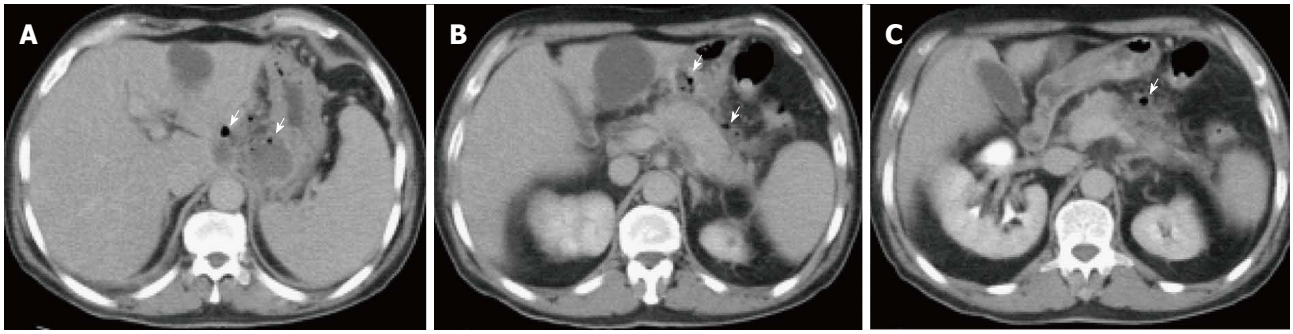

Figure 15 Infected peripancreatic fluid in a patient with acute pancreatitis. A-C: Axial CT scan during the portal venous phase. There are a few gas bubbles seen within a small peripancreatic fluid (arrows, A-C). In the absence of any intervention in keeping with infected peripancreatic fluid.

with necrosis and subacute pancreatic necrosis.

On CECT, walled-off necrosis demonstrates a heterogeneous fluid and non-fluid attenuation with varying degree of loculations surrounded by a well-defined and enhancing encapsulating wall; which may involve both the pancreatic and extrapancreatic tissue. CECT, however, may not readily distinguish solid from fluid contents; as a result, pancreatic and peripancreatic necrosis may be misdiagnosed as a pancreatic pseudocyst. For this purpose, MRI may be required for this distinction (Figure 14).

Infected pancreatic necrosis

Pancreatic and peripancreatic necrosis can remain sterile or become infected. The development of secondary infection in pancreatic necrosis is associated with increased morbidity and mortality[3]. Most studies suggest that there is no absolute correlation between the extent of necrosis and the risk of infection and duration of symptoms[7]. The early diagnosis of infected pancreatic necrosis is very important in the initiation of antibiotic therapy.

The diagnosis of infected ANC or WON can be suspected in the presence of extraluminal gas on CT or MRI. This extraluminal gas is present in areas of necrosis and may or may not form a gas/fluid level depending on the amount of fluid content present at that stage of the disease (Figure 15). The diagnosis may be confirmed by aspiration and analysis including microscopy and culture.

SEVERITY OF ACUTE PANCREATITIS

Clinical vs MCTSI vs MRSI severity index

Several clinical scoring systems like Marshal, SOFA. APACHE or Ranson criteria were designed to accurately correlate the complications like organ failure and mortality in acute pancreatitis. In the last two decades, radiological scoring systems were developed to accurately diagnose and correlate complications in acute pancreatitis.

For the first time in 1990, Balthazar *et al*[28] introduced the CT severity index for assessment of AP; which correlated well with morbidity, mortality and length of hospital stay. CTSI was widely adopted in clinical and research settings; however, a potential limitation was its inability to detect pancreatic necrosis. MCTSI introduced by Mortele *et al*[39] in 2004 to account for the limitations of CTSI (Table 2); which showed improved correlation with severity.

MCTSI incorporated extrapancreatic manifestations and simplified the evaluation of extent of parenchymal necrosis by categorizing into none, less than 30% or more than 30%; in addition to evaluating peripancreatic inflammation by detecting the presence or absence of

Table 2 MCTSI scoring ystem[39]

Prognostic Indicators	Characteristics	MCTSI[1]
Pancreatic inflammation	Normal pancreas	0
	Pancreatic ± peripancreatic inflammatory changes	2
	One or more collection or peripancreatic fat necrosis	4
Pancreatic necrosis	No necrosis	0
	< 30%	2
	> 30%	4
Extrapancreatic complications (pleural effusions, ascites, vascular, gastrointestinal, *etc.*)		2

[1]Scores ⩾ 5 are associated with higher morbidity and mortality.

Table 3 MR severity index scoring system[69]

Prognostic Indicators	Characteristics	MRSI
Pancreatic inflammation	Normal pancreas	0
	Focal or diffuse enlargement of the pancreas	1
	Intrinsic pancreatic abnormalities with inflammatory changes in the peripancreatic fat	2
	Single, poorly defined fluid collection	3
	Two or more poorly defined collection or presence of gas in or adjacent to the pancreas	4
Pancreatic necrosis	No necrosis	0
	< 30%	2
	30%-50%	4
	> 50%	6

peripancreatic fluid. Predictive accuracy of CT scoring systems for severity of AP and comparisons between CTSI and MCTSI were made[40]. They reported that they could not detect any significant differences between CTSI and MCTSI in evaluating the severity of AP. Their study also demonstrated that compared with APACHE II, both CT indexes more accurately diagnosed clinically severe disease and correlated better with the need for intervention and pancreatic infection.

It has been reported that MR severity index (MRSI) significantly correlated with CTSI (Table 3), Ranson score, C-reactive protein levels, appearance of systemic complications, duration of hospitalization and clinical outcome[17,41].

Chronic pancreatitis

Chronic pancreatitis is defined pathologically by continuous or relapsing inflammation of the organ leading to irreversible morphologic injury and typically leading to permanent impairment of both exocrine and endocrine functions. The incidence of chronic pancreatitis ranges from 5-12 per 100000 people in industrialized countries[1].

Table 4 Imaging criteria for chronic pancreatitis[70]

	CT criteria	MRI/S-MRCP criteria
Moderate chronic pancreatitis	⩾ 2 of the following:	Moderate pancreatogram changes
	Main duct enlarged (2-4 mm)	Main duct abnormal and
	Slight gland enlargement (up to 2 × normal)	Abnormal side branches, > 3
	Heterogeneous parenchyma	
	Small cavities (< 10 mm)	
	Irregular ducts	
	Focal acute pancreatitis	
	Increased Density of the main pancreatic duct wall	
	Irregular head/body contour	
Marked chronic pancreatitis	with ⩾ 1 of the following	Main duct abnormal and
	Large cavities (> 10 mm)	Abnormal side branches, > 3
	Gross gland enlargement (2 × normal)	Plus one or more of the following:
	Intraductal filling defects or pancreatic calculi	Large cavity
	Duct obstruction, stricture, or gross irregularity	Obstruction
	Contiguous organ invasion	Filling defects
		Severe dilatation or irregularity

Chronic pancreatitis is a cause of abdominal pain, weight loss, steatorrhea and diabetes mellitus, which may occur as a consequence of multiple factors, including biliary stone disease, alcohol consumption, malignancy, metabolic disorders, and various genetic and environmental insults, including trauma[1].

The histopathological changes in chronic pancreatitis evolve from unevenly distributed fibrosis in early chronic pancreatitis to diffuse fibrosis involving the entire gland in late stages. In advanced disease, large areas of acinar parenchyma are replaced with sclerotic tissue causing atrophy. Ductal irregularities like strictures, dilatation and side branches ectasia occur due to surrounding fibrosis. Other characteristic findings of severe chronic pancreatitis are calcifications and presence of complications like pseudocyst, vascular aneurysms and venous thrombosis.

Role of imaging

Imaging plays a significant role in detecting parenchymal and ductal abnormalities in chronic pancreatitis and helps in differentiating early from advanced phases to a certain extent; which further guides the management of these patients.

Most commonly accepted CT- and MRI-based criteria for diagnosis of chronic pancreatitis are shown in Table 4.

Early chronic pancreatitis

Ultrasound and CT are insensitive in diagnosis of early chronic pancreatitis, as they often show no abnormalities. A recent study showed that parenchymal changes

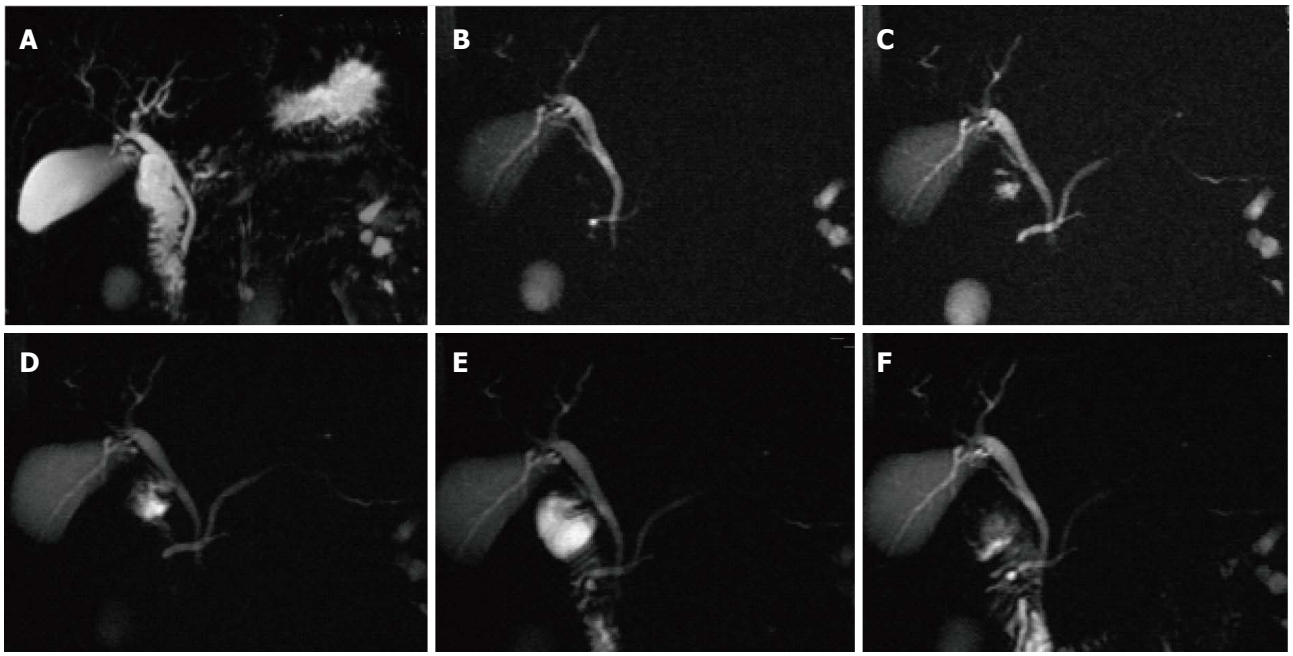

Figure 16 Pancreatic divisum, with a small Santorinicele. A: Coronal 3D- maximum intensity projection MRCP image before administration of secretin; B-F: Selected dynamic secretin thick-slab MRCP images obtained at 30 s (B), 60 s (C), 120 s (D), 4 min (E) and 9 min (F). Prior to administration of secretin, it is difficult to identify the main pancreatic duct (A). After administration of secretin, there is better delineation of the main pancreatic duct (C), with demonstration of pancreatic divisum. There is also enlargement of the accessory pancreatic duct, with demonstration of a small santorinicele (B, F). S-MRCP allows qualitative and quantitative assessment of pancreatic exocrine secretions. In this case, the pancreatic flow output was considered within normal limits; excluding early chronic pancreatitis.

might precede ductal changes in chronic pancreatitis; thus depicting the importance of MRI compared to MRCP in early diagnosis of disease[42].

On MRI, normal pancreas is hyperintense on T1 weighted images and shows uniform enhancement on the late arterial phase (Figure 1). MRI detects not only morphologic characteristics, but also early fibrotic changes. Fibrosis is shown by diminished signal intensity on T1-weighted fat-suppressed images and diminished enhancement on immediate post-Gadolinium gradient-echo images[43]. Low signal intensity on fat-suppressed T1-weighted images reflects loss of the aqueous protein in the acini of the pancreas. Diminished enhancement on capillary phase images reflects disruption of the normal capillary bed and increased chronic inflammation and fibrosis.

MRCP findings in early chronic pancreatitis often demonstrate normal main pancreatic duct with dilated and irregular side duct branches. The limiting factor is the underestimation of ductal size. Some investigators reported that patients with abnormal MR imaging findings but normal MRCP might benefit from dynamic secretin-MRCP (S-MRCP) (Figure 16); which may reveal ductal abnormalities due to improved visualization otherwise not detected on MRCP[42]. Secretin-MRCP has been reported to show ductal changes, like dilatations and strictures in early chronic pancreatitis.

EUS has a prominent role in chronic pancreatitis for its ability to detect early morphologic changes. Endoscopic retrograde cholangiopancreatography (ERCP) is considered to be gold standard test in detecting early changes, but unlike ERCP, EUS is relatively a non-invasive procedure, and also helps in the evaluation of both pancreatic duct and parenchymal changes compared to ERCP that has limitation in evaluating pancreatic side branches and parenchyma[44]. Chong *et al*[45] showed sensitivity of 83% and specificity of 80% of EUS for the diagnosis of chronic pancreatitis.

Late chronic pancreatitis

CT is reported to be 60% to 95% sensitive in diagnosing advanced disease as it can readily detect parenchymal changes associated with advanced chronic pancreatitis[46]. Most common findings on CT include dilatation of main pancreatic duct and its side branches; which can be seen in 68% of patients. The ductal contour may be smooth, beaded or irregular[47].

Other findings include intraductal calcifications, which is the most specific finding and is seen in nearly half of the patients with chronic pancreatitis and parenchymal atrophy (Figure 17). However, parenchymal atrophy is neither specific nor sensitive as it seen normally with aging. Intraductal or parenchymal calcifications are usually seen with alcohol related chronic pancreatitis but not on chronic pancreatitis resultant from other causes.

All patients with late or advanced chronic pancreatitis show diminished signal intensity of the pancreas on T1-weighted fat-suppressed images, an abnormally low percentage of contrast enhancement on immediate post-contrast images, and progressive parenchymal enhancement on the 5-min delayed post-contrast images; reflecting the pattern of enhancement of fibrous tissue.

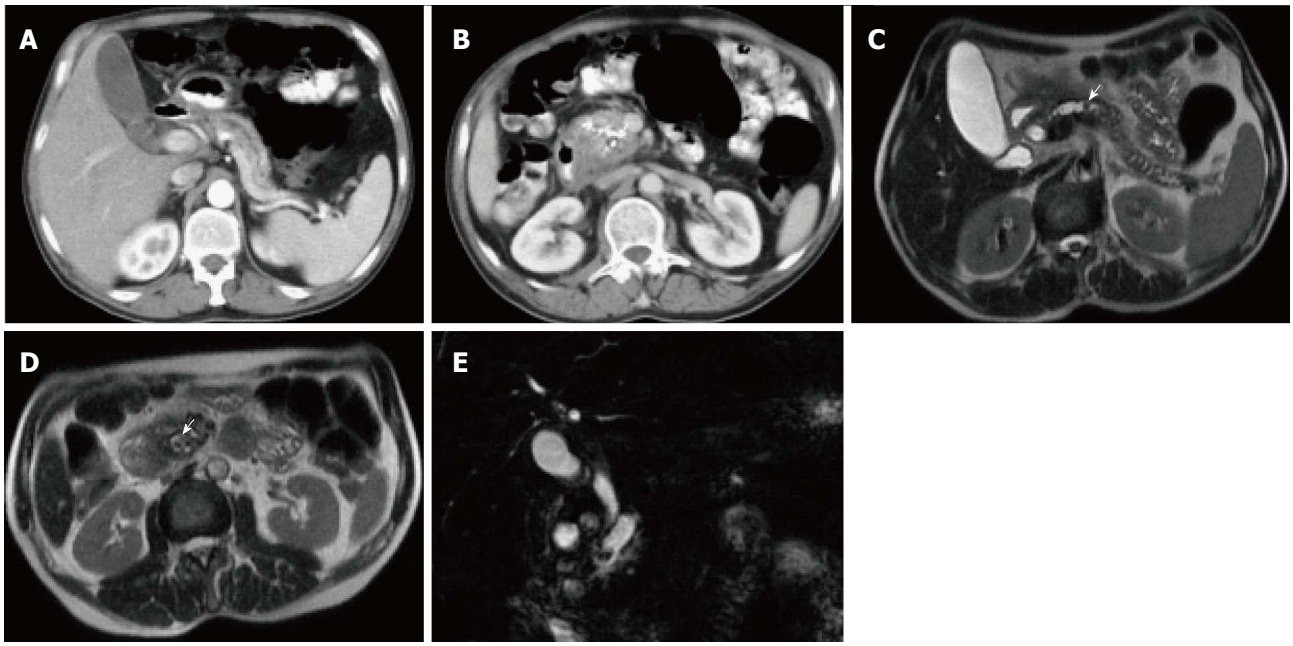

Figure 17 Chronic pancreatitis with pancreatic parenchymal calcifications and pancreatic duct stones. A, B: Axial CT scan during the late arterial phase; C, D: Axial T2 single-shot fast spin-echo images; E: Coronal 3D- Cholangiopancreatogram (MRCP) image. CT shows a markedly dilated and tortuous main pancreatic duct (MPD) (A, B), with foci of thick calcification involving the pancreatic head and uncinate process parenchyma (B). Large the proximal MPD stone was suspected on CT (arrow, B). MRCP shows gross pancreatic ductal dilatation with confirmation of the distal intraductal calculus (arrow, D), and shows an additional mid-pancreatic duct stone, not clearly seen on CT (arrow, C). No pancreatic masses or ductal anomalies are identified.

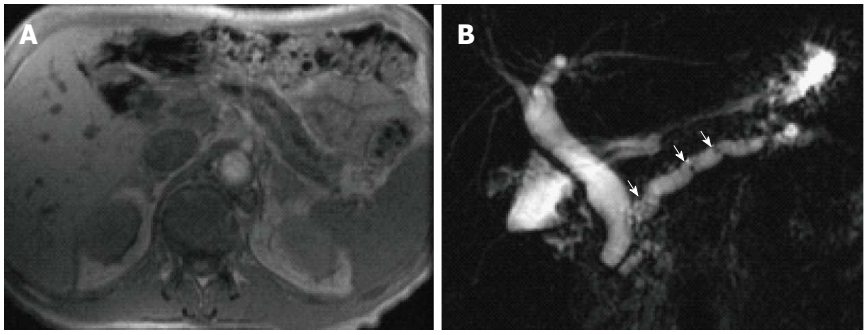

Figure 18 Chronic pancreatitis. A: Axial T1-weighted GRE MRI. B: Coronal-oblique thick-slab MRCP image. There is evidence of diffuse thinning of the pancreatic parenchyma with uniform dilatation of the pancreatic duct and prominence of the pancreatic duct side-branches (A-B), associated with multiple tiny stones at the proximal pancreatic duct (arrows, B) in keeping with chronic pancreatitis. There is also mild uniform dilatation of the CBD, which tapers down to the level of the pancreatic duct (B).

MRCP in advanced phase demonstrates dilatation of the main pancreatic duct with ectasia of the side branches (Figure 18); giving chain of lakes appearance manifested as pancreatic ductal strictures, irregularities and intraductal calculi, appearing as hypointense filling defects.

Enlarged pancreatic head in chronic pancreatitis vs adenocarcinoma

Chronic pancreatitis may involve only the pancreatic head in 30% of patients, resulting in focally enlarged pancreatic head. In these cases, the focus of chronic pancreatitis can simulate the appearance of pancreatic ductal adenocarcinoma.

Both chronic pancreatitis and adenocarcinoma show similar imaging characteristics on CT and MRI due to abundant fibrosis and ductal obstruction; therefore, making the differentiation between these two entities very difficult. Both are generally seen as hypodense lesions on CT, mildly hypointense on T1-weighted images and heterogeneously mildly hyperintense signal on T2-weighted images. However, certain imaging characteristics are helpful in distinguishing enlarged pancreatic head in chronic pancreatitis from adenocarcinoma (Table 5).

Rarely, chronic pancreatitis may involve only the focally enlarged portion of the pancreas, with the reminder of the pancreas having no inflammatory changes. In these cases, the focus of chronic pancreatitis can also simulate the appearance of pancreatic ductal adenocarcinoma. The inflammatory process may also be sufficiently

Table 5 Differentiating imaging features between chronic pancreatitis and pancreatic adenocarcinoma

Chronic pancreatitis	Pancreatic adenocarcinoma
Preserved glandular, feathery or marbled texture similar to that of the remaining pancreas	Definable, circumscribed mass lesion is most often diagnostic for tumor, which disrupts the underlying architecture and results in loss of anatomic detail
Heterogeneous pancreatic enhancement with presence of signal void (cysts and calcifications) on immediate post-gadolinium images	Irregular, heterogeneous, diminished enhancement on postgadolinium images compared to adjacent pancreatic parenchyma
Irregular dilatation of main pancreatic duct with gradual narrowing	Abrupt cut off of the pancreatic duct with significant proximal dilatation +/- presence of double duct sign
Presence of multiple intraductal calcifications (the most specific finding)	Very few ductal calculi compared to chronic pancreatitis
Dilatation of main pancreatic duct with and ectasia of the side branches, giving chain of lakes appearance	Minimal dilatation of side branches
No vascular encasement, significant lymphadenopathy or distant metastasis	Vascular encasement, lymphadenopathy or distant metastasis

destructive that underlying stromal pattern is lost. In these rare cases, diagnosis can only be established by surgical resection and histopathological examination to confirm the absence of malignancy.

Despite the high-resolution images produced by conventional EUS, there are no specific EUS imaging features that can differentiate pancreatic cancer from other common mimics, including lymphoma, focal pancreatitis, neuroendocrine tumors, metastases, and focal AIP[48]. However, one of the strengths of EUS is its ability to allow guided fine needle aspiration (FNA); which may overcome this problem.

In a retrospective analysis by Agarwal *et al*[49], 110 patients with abnormal CT or MRI with an enlarged head of the pancreas or dilated pancreatic duct with or without dilation of the common bile duct underwent EUS or EUS-FNA. The study revealed an accuracy of 99.1% for EUS and/or EUS-FNA in diagnosing pancreatic neoplasm with a sensitivity of 88.8% and specificity of 100%[49]. Given the high accuracy in the evaluation of pancreatic tumors, Eloubeidi *et al*[50] proposed routine EUS-FNA for the differential diagnosis of solid pancreatic masses. Other studies have shown that a negative EUS in ambiguous cases (where a mass is suspected) has a high negative predictive value[51,52].

Positron emission tomography-computed tomography (PET-CT) has an established role in the diagnosis of pancreatic carcinoma, especially when cross sectional imaging or biopsies are equivocal or nondiagnostic. In patients with a suspicion of pancreatic malignancy, a focal increase in ^{18}F-fluorodeoxyglucose (FDG) uptake suggests the diagnosis of malignancy. Nonetheless, the cutoff value of maximum standardized uptake value (SUVmax) is not defined, as it overlaps in benign and malignant pancreatic disease processes[53,54].

Furthermore, FDG-PET's detectability of pancreatic cancer depends on lesion size and degree of FDG uptake and surrounding background uptake. In the setting of chronic pancreatitis, FDG-PET is shown to detect pancreatic adenocarcinoma with a sensitivity of 92% and with a negative predictive value of 87%. In the setting of acute pancreatitis, the specificity can be as low as 50%, as it is known that inflammatory tissue can also demonstrate FDG activity[47].

Complications of chronic pancreatitis

The most common non-neoplastic complications of chronic pancreatitis include pseudocysts, pseudoaneurysms (due to erosion of the arterial wall), splenic vein thrombosis with subsequent development of collaterals, biliary obstruction (due to pseudocysts), and gastrointestinal complications like gastric outlet obstruction or bowel ischemia[6,55]. These complications are well depicted with CT and MRI.

MRI with MRCP may be superior to CT in detecting specific complications like pseudocysts, fistula formation, distal common biliary dilatation and vascular complications associated with higher morbidity and mortality[46].

Special types of chronic pancreatitis autoimmune pancreatitis.

Autoimmune pancreatitis is a distinct form of pancreatitis characterized clinically by obstructive jaundice (with or without pancreatic mass), histologically by a lymphoplasmacytic infiltrate and fibrosis and therapeutically by a dramatic response to steroids[56].

Autoimmune pancreatitis accounts for 2%-6% of chronic pancreatitis[57,58]. It is associated with other autoimmune disorders like Sjogren's syndrome, primary biliary cirrhosis, and primary sclerosing cholangitis[59,60]. Early diagnosis of autoimmune pancreatitis is crucial as it often responds to steroid therapy; thus avoiding complications.

In AIP, affected areas appear enlarged and hypodense on CT. CECT demonstrates diminished enhancement of the involved parenchyma on the late arterial phase and delayed enhancement on the delayed phase (Figure 19). The MR appearance of autoimmune pancreatitis is similar and is characterized by enlarged pancreas with moderately decreased signal intensity on T1-weighted images, mildly high signal intensity on T2-weighted images and delayed post-gadolinium enhancement of the pancreatic

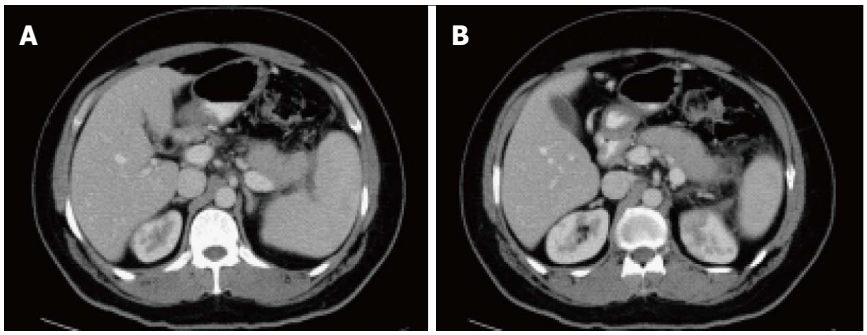

Figure 19 Autoimmune pancreatitis. A, B: Axial CT scan during the late arterial phase. There is evidence of diffuse pancreatic swelling with loss of the normal pancreatic lobulation, obliteration of the pancreatic duct and subtle low attenuating peripancreatic rim (A, B) in keeping with autoimmune pancreatitis. Patient had high IgG4 level (> 0.500 g/L).

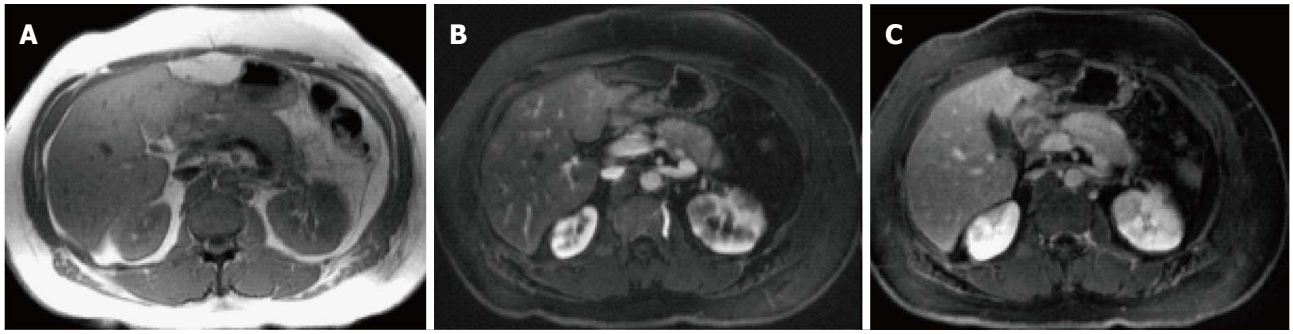

Figure 20 Autoimmune pancreatitis. A: GRE T1-weighted image; B, C: Post- contrast 3D-GRE T1-weighted images with fat-suppression during the late arterial and portal venous phases. There is evidence of diffuse pancreatic swelling with reduced T1 signal, loss of the normal pancreatic lobulation and obliteration of the pancreatic duct, associated with a rim of low T1 signal (A). The pancreas demonstrates diffuse reduced enhancement on the late arterial phase and progression of enhancement on the portal venous phase in keeping with autoimmune pancreatitis. The patient had significant biliary tree irregularities in keeping with primary sclerosing cholangitis (not shown). Additionally, there are a few bilateral wedge-shaped areas of renal hypo- enhancement in keeping with segmental infarcts.

parenchyma (Figure 20). Additional findings that may be observed in autoimmune pancreatitis include: (1) capsule like rim surrounding the diseased parenchyma, that is hypointense on T2-weighted images and may show delayed post-gadolinium enhancement[59]; (2) absence of parenchymal atrophy; (3) ductal dilatation proximal to the site of stenosis; (4) absence of peripancreatic fluid; and (5) clear demarcation of the abnormality[60].

MRCP depicts diffuse or segmental narrowing and irregularity of the main pancreatic duct as characteristic findings. The most commonly involved segment is the intrapancreatic common bile duct, and less frequently multifocal intrahepatic biliary strictures are noted.

Autoimmune pancreatitis is has 3 types based on morphologic patterns: diffuse, focal, and multifocal. Diffuse disease is the most common type. CT and MRI commonly show a swollen, sausage-like pancreas with poorly demonstrated borders and a capsule-like rim of low-density/intensity[61].

The diffuse form of AIP may mimic diffuse disorders like lymphoma, metastases or other diffuse infiltrative processes. In most of these disorders, unlike AIP, the parenchyma is heterogeneous and shows irregular contours.

Focal disease is less common and manifests as a well-defined hypodense mass, often involving the head and mimicking pancreatic adenocarcinoma. In patients who underwent pancreatic resection for suspected malignancy, 2.5%-8% were ultimately diagnosed with AIP without malignancy[58,62]. However, the probability of AIP *vs* pancreatic cancer in patients with obstructive jaundice can be predicted based on CT/MRI findings.

Diffusely enlarged pancreas showing low density mass with enhancement on delayed phases on CT/MRI, especially with a capsule-like rim, and no pancreatic ductal cutoff is highly likely to have AIP. Low-density mass on CECT, pancreatic ductal cutoff in presence or absence of pancreatic atrophy mostly suggests pancreatic cancer.

Groove/paraduodenal pancreatitis

Groove pancreatitis is a rare form of focal chronic pancreatitis involving the anatomic groove between the pancreatic head, duodenum and common bile duct. Groove pancreatitis is categorized into 2 forms: pure, involving exclusively the groove; and segmental, involving the groove and extending in to the pancreatic head[63] (Figure 21).

Pathogenesis remains controversial but may result from obstruction of the accessory pancreatic duct as it drains into the second portion of the duodenum through the

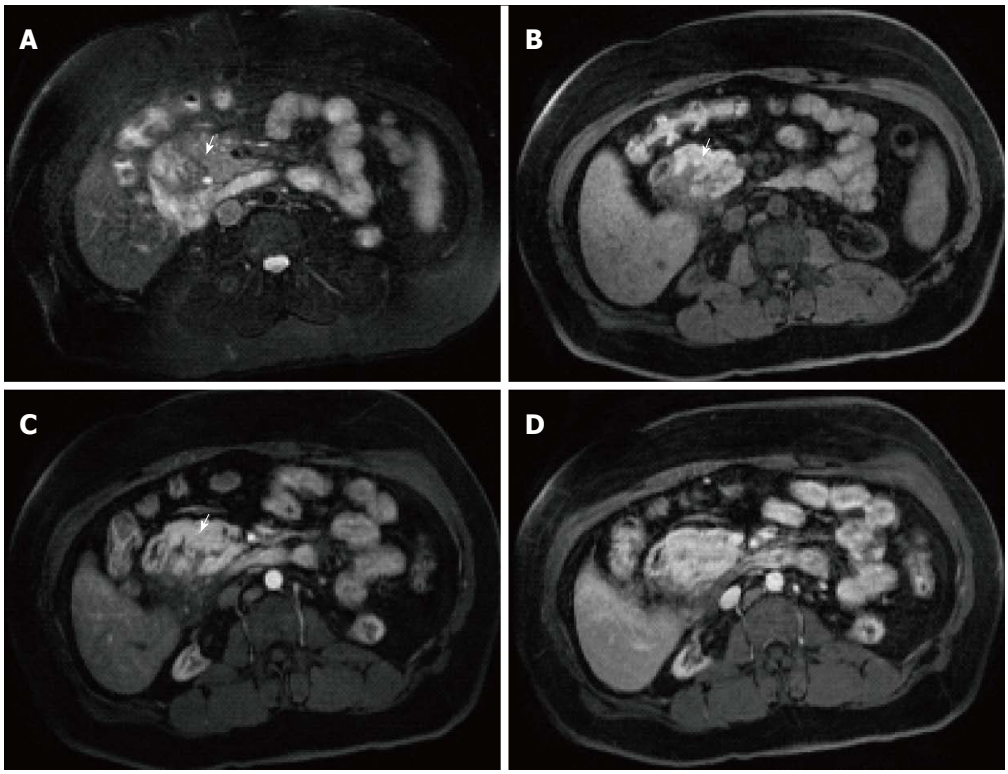

Figure 21 Groove pancreatitis. A: Axial T2-weighted single-shot fast spin-echo (SS-FSE) images with fat-suppression; (B) Pre- and (C, D) Post-contrast 3D-GRE T1- weighted images with fat-suppression during the late arterial and portal venous phases. There is a slightly low T2 signal sheet-like mass in the pancreaticoduodenal groove, with tiny cystic changes (arrow, A). The mass shows low T1 signal with extension into the pancreatic head (arrow, B). Imperceptible enhancement is depicted on the immediate post-contrast image (arrow, C), with progressive enhancement on the subsequent delayed images (D) in keeping with groove pancreatitis.

minor ampulla[64]. Presence of cystic changes, frequently located in the expected region of the pancreatic accessory duct, is considered a prominent feature of this process, likely related to accessory duct obstruction[65]. It is commonly seen in patients with history of alcohol abuse[64].

The classic MDCT features in the pure form can range from ill-defined fat stranding to frank soft tissue within the pancreaticoduodenal groove with increased delayed enhancement due to fibrosis. Thickening of medial duodenal wall on coronal images and presence of cysts can be appreciated sometimes[66]. On MRI, groove pancreatitis is characterized by a sheet-like mass in the groove that shows low signal on T1-weighted images, slightly high signal on T2-weighted images relative to the pancreas and may show delayed enhancement. Cystic lesions are well shown on T2-weighted images in the groove or duodenal wall[63].

It may be challenging to differentiate groove pancreatitis from pancreatic head duct adenocarcinoma. Recently, it was shown that by using three strict diagnostic criteria for groove pancreatitis: (1) focal thickening of the second portion of the duodenum; (2) abnormal increased enhancement of the second portion of the duodenum; and (3) cystic changes in the region of the pancreatic accessory duct, distinction from pancreatic duct adenocarcinoma could be achieved with high diagnostic accuracy (87.2% of patients), and a diagnosis of cancer could be excluded with a negative predictive value of 92.9%[67].

Hereditary pancreatitis

Hereditary pancreatitis is an autosomal dominant disease presenting as multiple episodes of pancreatitis in the absence of any predisposing factors. Imaging findings include parenchymal and intraductal calcifications and parenchymal atrophy. However, in hereditary pancreatitis, imaging plays an important role to rule out structural causes of pancreatitis and to closely monitor the development of pancreatic cancer, the risk of which is increased by many folds in these patients.

CONCLUSION

In summary, imaging plays an important role in the diagnosis and staging of acute and chronic pancreatitis. Both CT and MRI are widely used and represent the best cross sectional techniques in the setting of pancreatitis. Wider availability and good image quality make CT the mostly used imaging technique; however, due to its nonionizing nature, unmatched soft tissue contrast and higher safety profile of intravascular contrast media make MRI particularly valuable in pregnant patients, patients with recurrent pancreatitis and patients requiring multiple follow up examinations. Also, early form of chronic pancreatitis and some specific types of chronic pancreatitis benefit from being imaged with MRI.

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REVIEW

New insights to occult gastrointestinal bleeding: From pathophysiology to therapeutics

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Abstract

Obscure gastrointestinal bleeding is still a clinical challenge for gastroenterologists. The recent development of novel technologies for the diagnosis and treatment of different bleeding causes has allowed a better management of patients, but it also determines the need of a deeper comprehension of pathophysiology and the analysis of local expertise in order to develop a rational management algorithm. Obscure gastrointestinal bleeding can be divided in occult, when a positive occult blood fecal test is the main manifestation, and overt, when external sings of bleeding are visible. In this paper we are going to focus on overt gastrointestinal bleeding, describing the physiopathology of the most usual causes, analyzing the diagnostic procedures available, from the most classical to the novel ones, and establishing a standard algorithm which can be adapted depending on the local expertise or availability. Finally, we will review the main therapeutic options for this complex and not so uncommon clinical problem.

Key words: Obscure gastrointestinal bleeding; Angiodysplasia; Wireless capsule endoscopy; Double balloon enteroscopy

Core tip: This is an invited in depth review of occult gastrointestinal bleeding, addressing its pathophysiology, diagnosis and treatment. Our paper tries to unify in one single manuscript all what a general gastroenterologist should know about those items. From the essentials of pathophysiology, we have tried to build a rational approach to those patients' management depending on the severity of the condition, proposing an evidence-based management algorithm.

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INTRODUCTION

Gastrointestinal bleeding is a term that includes any bleeding originating from the esophagus to the anus. Classically, it has been classified in upper or lower depending on the location of the bleeding source, proximal or distal to the angle of Treitz.

The usual management of gastrointestinal bleeding (GIB) involves an upper endoscopy and colonoscopy in a first attempt to find the bleeding lesion. If those are unsuccessful, and there's a bleeding persistence or recurrence, the entity is called gastrointestinal bleeding of obscure origin or obscure gastrointestinal bleeding (OGIB), being the source of bleeding usually located in the small bowel. This seg-

Table 1 Causes of obscure gastrointestinal bleeding (in order of frequency)

Overlooked lesions in the upper GI tract or in the colon
Upper GI tract (proximal to the angle of Treitz)
Cameron ulcers
Fundic varices
Peptic ulcer
Angiectasia
Dieulafoy lesion
Gastric antral vascular ectasia
Colorectal lesions
Angiectasia
Polyps
Neoplasms
Anal disease
Dieulafoy lesion
Mid-GI tract lesions
< 40 yr
Meckel diverticulum
Dieulafoy lesion
Tumors (GIST, Lymphoma, Carcinoids, *etc.*)
Inflammatory bowel disease
Celiac disease
40-60 yr
Small bowel tumors
Angiodysplasia
Celiac disease
NSAID's related lesions
> 60 yr
Angiodysplasia
Small bowel tumors
NSAID's related lesions
Rare causes (< 1%)
Haemobilia
Aortoenteric fistula
Hemosuccus pancreaticus

GI: Gastrointestinal; NSAID: Non-steroidal anti-inflammatory drug.

ment of the gastrointestinal tract has been impossible to endoscopic exploration for a long time. It has been studied with suboptimal procedures such as small bowel series or enteroclysis in mild cases, or with more aggressive methods in severe cases, such as intraoperative enteroscopy (IE).

But the development of new endoscopic procedures like wireless capsule endoscopy or therapeutic procedures like the new enteroscopes, with different modalities of overtubes and balloons, has allowed an accurate exploration of this part of the GI tract, modifying significantly OGIB patients' management.

From 2006 a new OGIB classification has been proposed, based on the segment of the GI tract where the bleeding source is located, which determines the needed procedures for its diagnosis and treatment. Indeed, upper gastrointestinal bleeding is defined as the one with a bleeding source proximal to the ampulla, therefore accessible to upper endoscopy; mid GI bleeding is established when the causative lesion is between the ampulla and the ileocecal valve. Finally, lower GI bleeding has a colorectal bleeding source accessible to colonoscopy[1].

Therefore, obscure OGIB can be defined as a persistent or recurrent GI bleeding without a bleeding source found after performing upper endoscopy and colonoscopy. OGIB comprises 5% of all GI bleeding cases, constituting a diagnostic and a therapeutic challenge, either because of the morbidity and mortality associated, as well as for the high consumption of health resources for its diagnosis and treatment[2].

In most of OGIB patients (75%), the bleeding source is located in the small bowel[3], being normally a mid-GI bleeding[4]. The rest of the lesions are usually in areas accessible to conventional endoscopy, but overlooked in previous endoscopic procedures.

OGIB refers to two different clinical situations, regarding the onset of the GI bleeding: (1) Obscure-occult GI bleeding refers to the patient with a GI bleeding detected by a positive occult blood fecal test, with or without iron depletion; (2) Obscure-overt GI bleeding, in which an evident GI bleeding is seen, in the form of melena or hematochezia[5]. This review addresses the diagnosis and management of patients with obscure-overt GI bleeding, with a special interest in the different available procedures, establishing a management algorithm.

ETHIOLOGY

Causes of OGIB include overlooked bleeding lesions by upper endoscopy or colonoscopy, as well as the ones that, after an exhaustive endoscopic study, are classified as mid-GI bleeding[6]. The causative condition of OGIB is highly determined by age, being tumors as lymphoma, carcinoids, and GIST more likely in patients of less than 40 years, and vascular lesions as angiodysplasia more usual in elder patients, comprising 40% of all cases[7]. Table 1 contains the main recognized causes of OGIB[5].

PATHOPHYSIOLOGY OF THE MOST USUAL CAUSES OF OGIB

Angiodysplasia

Angiodysplasia is one of the most usual causes of over OGIB in patients older than 40 years, and the most frequent cause in patients older than 60 years[7]. They are also known as arteriovenous malformations or vascular ectasia, more frequently found in the stomach, duodenum, cecum and ascending colon. Most of them are acquired but some may be present at birth, or as a part of some hereditary syndromes[8]. Pathogenesis is uncertain and four theories have been proposed: (1) Some attribute angiodysplasia to a mild chronic venous obstruction. This hypothesis is concordant with the observation of a higher number of these lesions in the right colon, where the wall tension is higher; (2) They could be a complication of mucosal chronic ischemia, which could appear in episodes of bowel obstruction or after tough straining when defecating; (3) Some authors think they could be a complication of local ischemia in patients with heart, vascular or lung disease[9]; (4) Some of them, usually in younger patients, could be congenital or associated to hereditary syndromes; (5) It has also been suggested a pathogenic relation between aortic ste-

nosis and angiodysplasia, caused by the haemodinamic abnormalities determined by the valvular disease (Heyde Syndrome)[10]. Therapy is controversial, but some studies have shown a reduction in bleeding episodes after valvular replacement; and (6) In terminal cardiac failure, left ventricular assisted devices have been associated with increased bleeding episodes from angiodysplasia. In these cases, pathogenesis seems related with anticoagulant therapy, vascular malformations, loss of activity of Von Willebrand factor and mucosal ischemia[11].

Small bowel tumors

Despite infrequent, GI bleeding is the usual clinical onset, being more frequent in benign tumors as leyomioma than in malignant lesions as leyomiosarcoma[12]. Bleeding is caused by erosion of the tumor surface or by the rupture of aberrant vascular structures within the lesion.

Meckel diverticulum

This is a relevant condition in patients of less than 25 years old. Despite rare, it is the most frequent congenital abnormality in the GI tract. They are caused by the incomplete obliteration of the vitelin duct during embryogenesis, which leads to the formation of a true small bowel diverticulum[13]. Meckel diverticulum has all the bowel wall layers, and in 12%-21% of cases it may contain ectopic tissues (gastric or duodenal mucosa or even pancreatic ducts). They are usually asymptomatic, but may also cause abdominal pain or OGIB. Bleeding is caused by an ulceration of the small bowel due to acid secretion by heterotopic gastric mucosa contained within the diverticular layers. Bleeding can be chronic and insidious, or acute and massive, but transfusion is hardly ever required. The main anatomical risk factor that makes bleeding more likely is diverticula size of more than 2 cm[14].

Dieulafoy's lesion

Etiology is unknown. Lesions are normally found in the proximal stomach, in the lesser curvature, near de esophago-gastric junction. It is usually a submucosal, dilated, aberrant vessel that erodes the overlaying mucosa without a previous ulcer[15]. This is caused by the lack of ramification of the submucosal artery which makes its diameter ten times the normal diameter of a mucosal capillary. Triggering causes are unclear and it usually appears in male patients with comorbidities such as cardiovascular diseases, arterial hypertension, chronic kidney disease, diabetes or alcohol abuse. It is important to mention that this lesion can be overlooked in an endoscopic exam[16], given that quite frequently the aberrant vessel cannot be seen unless it bleeds actively.

Celiac disease and inflammatory bowel disease

GI bleeding is usually associated to complications in both conditions, which can be ulcers or tumors like adenocarcinoma or lymphoma.

At lasts, we would like to emphasize three rare OGIB causes, associated to a high mortality and a difficult diagnosis[17].

Haemobilia: It consists in the bleeding from the biliary tree caused by a communication with vascular structures. The most frequent causes are a closed traumatisms, hepatic artery or portal vein aneurisms, liver abscesses, neoplasms or secondary to procedures such as liver biopsy or bile duct stones extraction[18]. Diagnosis is always difficult[19]. It should be suspected in the anamnesis, when the patient presents upper right quadrant pain, jaundice and OGIB, but this is an unusual form of presentation. Diagnosis can be confirmed by direct endoscopic visualization of blood passing through the papilla. Angiography is a therapeutic option but, despite a successful embolization or surgical treatment of the originating vessel, mortality is high.

Aortoenteric fistula: It is an exceptional but severe cause of OGIB, usually related to a previous aortoiliac surgery. The most common cause of primary aortoenteric fistula is an arteriosclerotic aneurism, infectious aortitis or tuberculosis[20]. The most common cause of secondary aortoenteric fistula is an abdominal vascular graft infected, usually some years after its positioning. Pathophysiology involves a graft and surrounding tissue infection of low aggressiveness, usually caused by *S. aureus* or *E. coli*, with causes erosion and communication between the graft and the lumen of the GI tract[21]. Other secondary causes are penetrating ulcers, tumor invasion of the aorta, trauma or radiation. The onset is usually a self-limited premonitory bleeding episode followed, days or weeks later, by a second episode typically massive and life-threatening.

Pancreatic hemosuccus: It is usually caused by the erosion of the splenic artery by a pseudocyst which causes a pseudoaneurysm communicated with the pancreatic duct. Suspicion is arisen by the observation of blood emerging from the ampulla, in a plausible clinical scenario. Angio-CT scan can be diagnostic. Angiography can help to establish a diagnosis, and it can be also therapeutic, but frequently surgery is needed for bleeding control[21].

OGIB AND ANTICOAGULATION

Oral hypercoagulation therapy has been described as a factor increasing OGIB incidence, worsening prognosis and changing management. In a 2014 study the risk of a severe bleeding episode increased up to 4%-23%, being higher when INR was above 4[22]. Despite this, anticoagulants did not seem to modify the type of lesion that caused the bleeding[23,24].

Risk factors associated with a higher bleeding risk in patients under oral anticoagulants therapy are: (1) Age: In patients older than 70 years annual bleeding risk is 3%; (2) A previous episode of GI bleeding or peptic ulcer increases the risk in up to 2.1% to 6.5%; (3) Co-

morbidities: Chronic kidney failure, diabetes, cardiac disease, alcohol abuse; and (4) Association with antiplatelet drugs.

Recently, some new anticoagulants have been developed with lower rates of intracranial bleedings[25] but with a likely increase in GI bleeding[26].

DIAGNOSTIC AND THERAPEUTIC PROCEDURES IN THE PATIENT WITH AN OVERT OGIB

For the evaluation of OGIB, particularly mid GI bleeding, angiography, gamma praphy and intraoperative enteroscopy have been classically performed. But the technological improvements with capsule endoscopy, CT-angiography and balloon assisted enteroscopy (BAE) have relegated the classical techniques to a second step and are nowadays used only in selected patients. Moreover, the diagnostic procedure selected in each case depends largely on different factors, as patient's symptoms, bleeding severity, as well as local expertise and availability, or the need of therapeutic procedures.

Repeated upper and lower endoscopy

Bleeding lesions within reach of upper endoscopy have been indentified in 10%-64% of patients who underwent push enteroscopy and in 24%-25% of patients who underwent BAE because of a suspected OGIB. Nevertheless, few missing lesions are found in lower enteroscopy, with about 7% of findings within the reach of a conventional colonoscope, usually in patients with a previous poor bowel cleansing or with profuse bleeding. In the previously mentioned study, repeated endoscopy (upper or lower) revealed overlooked lesions in 15% of patients[27-32]. However, in another Australian paper, only 4% of 50 patients submitted for enteroscopy had overlooked lesions by upper or lower endoscopy, concluding that repeated endoscopy is not cost-effective[33].

Therefore, despite lesions within the reach of conventional endoscopy might be overlooked, it is not recommended to repeat these procedures in all cases, because this would raise the costs, delaying the definitive diagnosis and overloading endoscopy units. So, it is only recommended to repeat these procedures in selected cases, as in those with previous suboptimal results due to a bad bowel cleansing or with a recurrent GI bleeding with a high suspicion of an upper GI tract origin. If hemobilia or hemosuccus are suspected, upper endoscopy with a duodenoscope is mandatory.

Some authors recommend that, if needed, the second conventional endoscopy should be performed with a push enteroscope, which would allow a deeper exploration in case no other lesions are found in the upper GI tract[34,35].

Small bowel series

Neither small bowel series nor enteroclysis have a diagnostic accuracy of more than 5% (22) and 21% respectively[36], with a particular lack of accuracy in flat mucosal lesions, as angiodysplasia, a frequent cause of bleeding cause in the small bowel. The development of capsule endoscopy and enteroscopy has limited its use to a few situations[25,37,38].

The development of other radiologic methods as CT or MRI enteroclysis with new multidetector equipment, offers higher diagnostic capabilities, even for flat vascular lesions[39].

CT angiography

It has been recently added to the diagnostic armamentarium for OGIB, with a reported sensibility and specificity of 79%-90% and 95%-99% respectively[40,41]. It detects bleedings of 0.3-0.5 mL/min with a diagnostic accuracy near 100%, having the advantages of its availability and non-invasiveness. Nevertheless it lacks therapeutic capabilities, requires radiation exposure and need intravenous contrast with a known association with nephropathy and allergic reactions.

For all those reasons, CT angiography should be considered as the first diagnostic procedure in patients with active bleeding and hemodynamic impairment, instead of other procedures with a longer duration as gammagraphy, or more invasive as arteriography, which should be reserved for therapeutic purposes in patients with an active bleeding in CT angiography.

Furthermore, CT angiography has shown its usefulness in patients with an intermittent OGIB and a normal endoscopic study, leading to the detection of unusual cases of OGIB, like stromal tumors up to 1-2 cm. It is also the first option in diverticular disease with an excellent accuracy when studying vascular abnormalities causing GI bleeding, like aortoenteric fistulae.

Gammagraphy

Gammagraphy consists in the injection of patient's red cells tagged with Tc99 that survive in the bloodstream up to 24 h, leading to the detection of GI bleedings even of a very low rate (> 0.1 mL/min)[42]. Both properties make the procedure highly sensitive but with poor specificity, finding positive results in around 45% of patients in different published series[43]. The use of colloidal-sulphur Tc99 determines a quicker exploration because there is no need to tag red cells, but it has a lower accuracy because of the quicker dilution of the isotope in the bloodstream.

The main drawback of gammagraphy is its low precision when locating the bleeding source in the bowel, which can be mistaken in up to 25% of patients[44]. For these reasons, as well as for the high rate of false positive and negative results and the lack of therapeutic abilities, this procedure has a very limited role in OGIB, sometimes only as a previous step to angiography[45].

Angiography

It has diagnostic and therapeutic capabilities. It needs higher bleeding rate than angiography (> 0.5 mL/min),

with a better performance in severe cases. However, it allows the diagnosis of non-bleeding lesions as angiodysplasia or tumors and, for this reason, its sensibility shifts between 30% and 47%[38,46], while its specificity is usually near 100%. Nevertheless, angiography is an invasive procedure with associated risks and complications in up to 9.3% of patients[47]. Therefore, it is considered a second line procedure, limited to clinical pictures in which a lesion is likely.

A provocative test, giving to the patient hypo coagulants drugs, fibrinolytic agents or vasodilators, has not shown an improvement on angiography accuracy[48].

As a therapeutic method, it allows the administration of vasoactive agents inside the responsible vessel or to perform an embolization with different substances, leading to bleeding resolution in up to 70%-100% of patients[49,50].

Wireless capsule endoscopy

Wireless capsule endoscopy (WCE) has been a great progress in small bowel examination, representing a safe and minimally invasive method for the diagnosis of OGIB.

In a 2010 systematic review, 66% of WCE indications were OGIB, with a diagnostic yield of 60.5%, being angiodysplasia the most frequent finding (50%), followed by ulcers (26.8%) and neoplasms (8.8%)[51]. Different studies have shown that WCE is more useful in overt OGIB than in occult OGIB[51-55]. Other factors related to an increase in WCE yield are[56]: (1) Performance within two weeks after the bleeding episode; (2) Hemoglobin< 10 g/dL; (3) Persistence of GI bleeding for more than 6 mo; and (4) More than one overt bleeding episode.

But WCE has other potential roles, as the detection of overlooked lesions on conventional procedures like upper endoscopy[56] or colonoscopy[57], assessing number, size and location of lesions for a better planning of the therapeutic procedure. Indeed, in a 2008 study[58] from our group, 30 patients with angiodysplasia found on CE were followed for one year, observing that patients with bigger angiodysplasia (> 1 cm) have a higher clinical impact (lower hemoglobin rates, higher transfusion requirements) and therefore higher needs of therapeutic interventions after WCE (75% *vs* 18.2%), which lead to lower rates of rebleeding. In conclusion, this paper found that angiodysplasia size (> 1 cm) and number (> 10) is related with a higher mortality (20% *vs* 4% and 25% *vs* 0% respectively).

When compared with push enteroscopy or small bowel series, WCE has proved to be superior in OGIB: In a metaanalysis published by Triester in 2005, diagnostic yield of WCE was 63% compared to 28% and 6% of push enteroscopy and small bowel series respectively. Another meta-analysis of the same year showed similar results[59-61].

Regarding other procedures, CE has shown a higher yield than angiography or CT-angiography but very similar to BAE, with the differences of its invasiveness and its ability to explore the whole small bowel in a single procedure[62-64].

This higher yield has shown to have a direct impact on management of two thirds patients with OGIB[65,66], as well as a high negative predictive value, with a rebleeding rate after a normal CE in the following 19 mo of 5.6%[67].

Therefore, CE is a first line procedure for OGIB management, although it is far from the ideal. Important disadvantages, like biopsy sampling, lack of therapeutic abilities, lack of a remote motion control, battery limitations etc. imply the need of other methods to manage those patients[68,69].

Anyway, significant research is being conducted in this field, with devices that will likely allow biopsy sampling, therapeutic interventions, real time motion control from outside the patient by means of magnetic fields control or articulated arms (Spider Pill), improvements in batteries durability etc. Some of those advances have already been incorporated, as bleeding lesions detection improvements by pattern differences in color wavelength (FICE-CE, Given Imagin)[70].

Enteroscopy

Enteroscopy allows the endoscopic observation of the small bowel beyond the angle of Treitz by means of an enteroscope.

Push enteroscopy: Until recently, push enteroscopy (PE) has been the standard of care in patients with OGIB, after a normal upper endoscopy and colonoscopy. It consists in the passage of an enteroscope by mouth, which makes possible the exploration of a variable length of the small bowel, ranging from 30-160 cm beyond the angle of Treitz[71]. PE permits only a partial vision of the small bowel, but its main indication is still OGIB, with a global diagnostic yield of 12%-80% and better results in overt OGIB.

In conclusion, PE has the advantage of its therapeutic capabilities but also the important drawbacks of a partial exploration of the GI tract and its invasiveness. Thus, it should be carefully used for previously identified lesions which are likely within the reach of this enteroscope[25,71-75].

Double balloon enteroscopy: Double balloon enteroscopy (DBE) has been a great improvement in small bowel exploration, because it provides a complete examination of the bowel lumen, as well as biopsy sampling and therapeutic abilities[76].

First described in 2001, it was widely available in 2004, consisting in an enteroscope with a balloon attached to its tip, as well as another balloon over an overtube. The alternative inflation and progression with the overtube and the balloon-enteroscope system provides a deeper progression through the small bowel, with a significantly increased mean bowel length explored as compared to PE[77,78].

The combination of lower and upper DBE grants

a visualization of the whole length of the small bowel, which is not always needed[79].

Diagnostic yield of DBE in OGIB ranges between 47%-80%[5], similar to that of WCE[58]. Its yield is increased when the procedure is performed within one month after the bleeding event.

Keeping in sight the similar diagnostic yield of WCE and DBE, they are actually considered complementary procedures[80], being WCE the first tool to be used, because of its lower cost, less invasiveness and higher availability. Information from WCE examination is helpful to decide between an upper or lower enteroscopy. In case we don't have a previous WCE, or if an upper and lower enteroscopy is needed, it is usually recommended to begin the endoscopic examination with the upper enteroscopy, because it is technically easier and has an increased likelihood of finding the causative lesion[81,82].

The main drawback of DBE is that a complete small bowel examination is not feasible in up to 29%[5], it needs sedation (usually under general anesthesia), its availability is limited to referral centers, and it has a prolonged examination time and other difficulties usually found in the lower approach due to poor bowel preparation, abdominal adhesions etc.

Nevertheless, DBE is a safe procedure, with less than 1% complications, being the most usual perforation (0.4%), pancreatitis (0.3%), and ileus[83,84]. Complications are not related to age, but with therapeutic maneuvers or anatomical abnormalities of the bowel (*i.e.,* previous surgeries, abdominal radiotherapy or intestinal lymphoma treated with chemotherapy)[6].

Other enteroscopies: Other enteroscopes used with overtube and balloons are: (1) Single balloon enteroscopy (Olympus Inc.): Exploration times and depth of insertion in small bowel enteroscopy are similar to these of DBE. In a 2010 paper[85] 100 patients were studied (50 patients with DBE and 50 with SBE) achieving DBE a higher number of complete enteroscopies when compared with SBE (66% *vs* 22%) and a higher number of findings. However, in this study, a system different from the original SBE (Olympus®) was used (Fujifilm®), having a different flexibility and balloon pressure. Later, Takano *et al*[86] had to stop prematurely a study comparing DBE with SBE, because of the differences in complete enteroscopies between both systems (57.1% *vs* 0%), finding no differences with regards to findings or complications[86]. Finally, in 2011 and 2012 two studies with 130 and 107 patients respectively[87,88] showed no differences between both systems regarding depth of insertion, complete bowel examination, complications and findings; (2) Spiral enteroscopy (Endo-Ease Discovery SB, Spirus Medical Inc.): The device includes an overtube with a helical portion which grasps the bowel folds, reaching as far as 200 cm beyond the angle of Treitz; and (3) Shapelock (USGI Medical Inc.): It consists in an overtube with multiple titanium rings, joined by four titanium wires and covered by a detachable sheath. When tension is applied on the wires, the overtube is fixed, allowing the passage of the enteroscope. Today, it has more applicability in patients with altered anatomy due to previous surgeries, in incomplete colonoscopy and in NOTES[89-92].

Intraoperative enteroscopy: It has been considered the gold standard for small bowel examination for long time[4], and although balloon assisted enteroscopy (BAE) is preferred because it is less invasive and have similar results in the diagnosis and management of small bowel disorders, the IE is an important reserve tool. It consists in the insertion of the endoscope through an enterotomy, exploring the mucosa while the surgeon facilitates the advance of the endoscope and observes the serosal surface. Palpation and transilumination play an important role in this procedure, which allows the whole bowel examination in more than 90% of patients.

Intraoperative enteroscopy (IE) has a diagnostic yield of 50%-100%[4,93], with therapeutic possibilities, but it is invasive. 12%-33%[77,78] of complications and 8%[94-96] of mortality limit its use to cases in which the other diagnostic methods are contraindicated or impossible, and always in patients with clinically significant OGIB[93-98].

PROPOSAL OF A DIAGNOSTIC ALGORITHM

In a patient with OGIB, after conventional upper endoscopy and colonoscopy, we should consider to repeat colonoscopy when a poor bowel cleansing is reported, or when we suspect an incomplete colonic evaluation. Upper endoscopy should be repeated if a strong suspicion of an upper GI tract bleeding lesion is still a concern despite a previous normal upper endoscopy.

Once a bleeding cause within reach to conventional endoscopy has been ruled out, depending on patient' s situation, an evaluation of the small bowel by WCE and BAE (balloon assisted enteroscopy, DBE or SBE) should be the next step, beginning with the less invasive one, which is WCE[5,67,75,99].

After normal WCE, if the bleeding stops spontaneously, a conservative attitude is recommended, with a clinical follow-up of the patient. If there is a strong suspicion of small bowel disease, even with a previous normal WCE, BAE should be performed[100].

Nevertheless, some authors think that if pretest likelihood of a correct diagnosis and treatment with BAE is higher than 25%-30%, we should proceed directly with BAE, because it is the most cost-effective option[101,102]. Moreover, in centers with a high number of patients and experienced endoscopists, DBE can be considered as a first step procedure in some clinical settings[102].

After rebleeding, repeated WCE finds lesions in up to 20%-35% of patients. Those lesions can be found and treated afterwards with BAE, which can also detect up to 30% of lesions previously overlooked by WCE[103-105].

If a patient presents hemodynamic instability and an active bleeding, angiography and IE should be the first

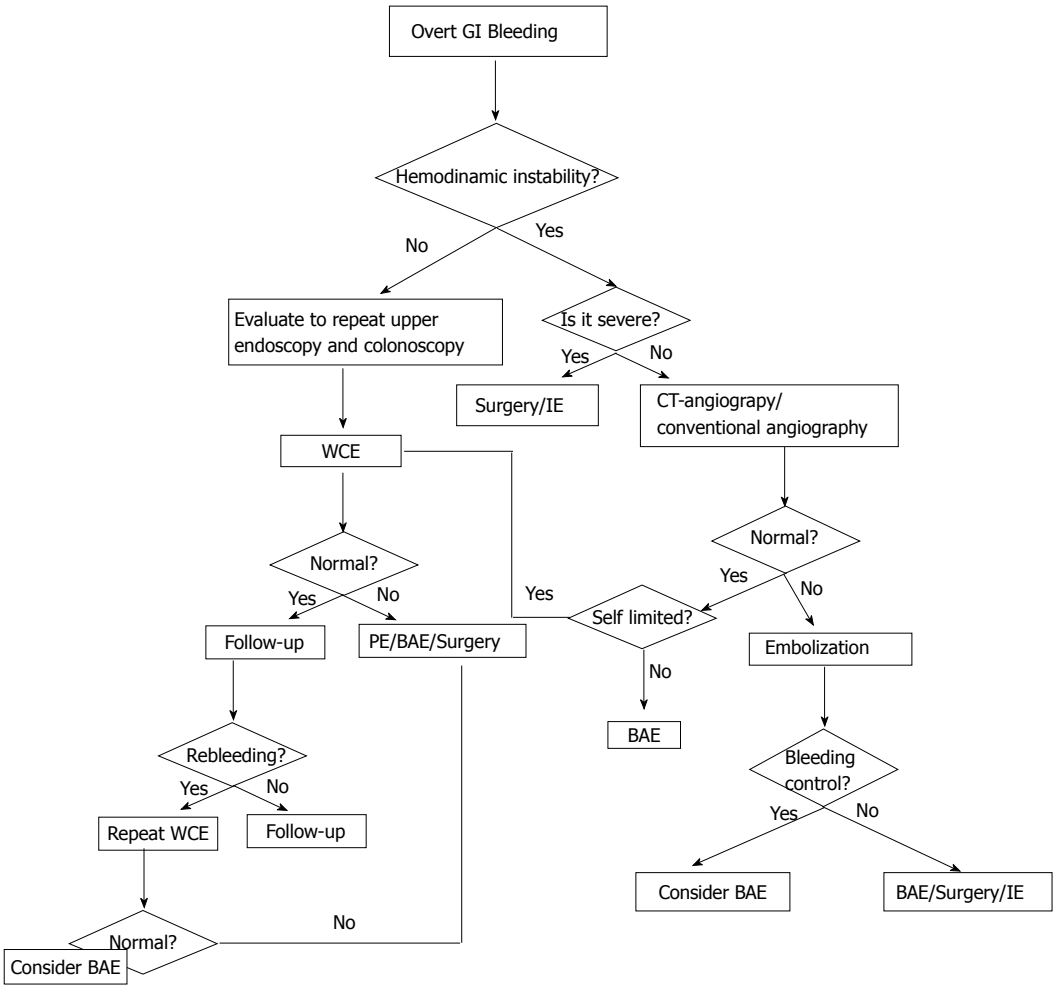

Figure 1 Proposal of a diagnostic algorithm. OGIB: Obscure gastrointestinal bleeding; WCE: Wireless capsule endoscopy; DBE: Double balloon enteroscopy; PE: Push enteroscopy; IE: Intraoperative enteroscopy.

diagnostic procedures. CT angiography is increasingly being used in this setting, because it offers an accurate diagnosis in many patients, it is less invasive, widely available and quick. Anatomical location of the lesion is usually accurate with few complications. After detecting a lesion by CT-angiography, conventional angiography or surgery can be used to apply the specific therapy[106] (Figure 1).

OGIB THERAPY

Therapy is directed by the type of lesion and its location. There are three major types of available therapies.

Pharmacological therapy[107]

Hormonal therapy (estrogens and progesterone) was initially explored by Koch et al in 1952 after observing that a patients with hereditary hemorrhagic theleangiectasia (HHT) whose bleeding varied depending on her menstrual cycle. The mechanism of action is not well understood, but there are several theories: (1) Estrogen and progesterone receptors have been detected in nasal and epidermal telangiectatic lesions in patients with HHT, and the hormone-receptor binding improved endothelial integrity in patients with HHT; (2) In animals this treatment improved vascular stasis within the mesenteric microcirculation and decreased the mucosal blood flow; (3) In patients on dialysis, estrogens shorten bleeding time by the reduction of endothelial prostacyclin production; and (4) Finally hormones may also decrease vascular endothelial growth factor.

Estrogens and progesterone therapy has been widely used in OGIB, with contradictory results, although some reports have observed a significant reduction in transfusion requirements, and even a complete resolution of bleeding.

A study of 43 patients, 38 of which were treated with hormonal therapy and followed for a mean time of 535 d (range 25-1551 d), reported benefits in patients with bleeding from sporadic angiodysplasia[108]. However, this has not been confirmed in other studies. The best data come from a multicenter, placebo-controlled trial involving 72 noncirrhotic patients which had bleedings from documented angiodysplasia; there was no benefit from hormonal therapy[109]. Based on these findings, hormonal therapy seems to have poor therapeutic advantages in

patients with sporadic angiodysplasia.

Some other papers do not recommend their use because of their lack of beneficial effects on OGIB and their adverse events (thrombosis, gynecomastia, loss of libido in males, metrorragia…). In general, their efficacy has not been proved, except for the treatment of hereditary hemorrhagic telangiectasia, von Willebrand disease, chronic kidney failure and gastric antral vascular ectasia (GAVE), in which hormonal therapy reduces transfusion requirements but not the size or number of lesions[98-100].

Somatostatin analogs: Octeotride reduces splacnic arterial flow by inhibiting angiogenesis and endothelial related growth factors[101-103]. Also, octeotride can inhibit angiogenesis by inhibiting endothelial cell proliferation. It has shown efficacy in acute and chronic GI bleeding, and can be used in patients with contraindications or a poor response to hormonal therapy. In Rossini et al study[110], treatment with octreotide in 3 patients decreased the need for blood transfusions during the follow-up period (8 to 17 mo). Other authors have published similar results[111], and have observed comparable side effects including diarrhea, steatorrhea, or changes in glucose metabolism.

A 2010 meta-analysis[112] analyzed 3 studies with a total of 62 patients, observing that 76% of patients responded to this therapy, achieving a significant reduction in transfusion requirements.

Depot formulations like LAR-Octeotride, which allow intramuscular administration once a month, have gained acceptance in selected cases[113,114]. In a study with 15 patients[115] treated with LAR-Octeotride for a recurrent bleeding from gastrointestinal angiodysplasia, the proportion of patients who experienced a bleeding event was lower during treatment than prior to treatment (20 *vs* 73), median transfusion requirements were reduced (2 *vs* 10 units), and median hemoglobin levels were higher during therapy (10 *vs* 7 g/dL).

Non-selective beta-blockers: They reduce splacnic flow, pulse and cardiac output. They are usually used in portal hypertension related OGIB and monotherapy or in association with LAR-Octeotride.

Thalidomide: It was retired in the 60s because of its teratogenic effect. However, thalidomide has recently shown to be an effective anti-inflammatory treatment in Crohn's disease. In addition to its anti-inflammatory effects, it also displays antiangiogenic activity, which may be useful for the treatment of GI bleeding. It can be taken orally and it could be used in patients with contraindications to other therapies. Obviously it is forbidden in childbearing aged women and in patients with peripheral neuropathy. It must be used cautiously in patients with cardiovascular or neurologic diseases, chronic kidney or liver failure and in immunosuppressed patients.

Some reports show promising outcomes in bleeding control[112]. In a randomized trial in 2011[116] patients treated with thalidomide were more likely than those treated with iron supplements to experience a positive clinical outcome (71% *vs* 4%).

Other drugs: (1) Antifibrinolitics: Tranexamic acid is an antifibrinolytic agent whose haemostatic effect is due to the inhibition of plasminogen activation in body fluids and tissues. Epsilon-aminocaproic acid has controlled chronic bleeding in patients suffering from HHT. These drugs have a prothrombotic activity and, for this reason, coagulation abnormalities or thrombophilia have to be ruled out before initiating the therapy; (2) Danazol: There are two single reports with positive results after hormonal therapy failure in patients with hereditary hemorrhagic teleangiectasia; (3) Desmopresin; and (4) Recombinant factor Ⅶ: Reserved to cases of massive overt OGIB.

Endoscopic therapy

There are different methods, injection therapies, thermal methods or mechanical devices which can be used with different endoscopes, depending on the location of the bleeding cause.

Argon plasma coagulation: It is safe and the most common and successful method used to treat angiodysplasia because of its easy application (especially for large superficial lesions), low cost, and reported limited depth of coagulation. Argon plasma coagulation (APC) has been used for a variety of bleeding lesions, including angiodysplasia, in these lesions submucosal saline injection prior to treatment with APC may protect against deep wall injury.

In a study of 50 patients with small bowel lesions, 44 patients were treated with APC for angiodysplasia[117]. After a mean follow-up of 55 mo, hemoglobin levels increased from a mean of 7.6 g/dL prior to treatment to 11.0 g/dL following it, and there was a significant decrease in the number of patients requiring blood transfusions. However, small bowel bleeding recurred in 21 of the patients treated with APC. A later study with 98 patients[118] reported similar results. The risk factors associated with rebleeding were the number of lesions and the presence of valvular and or arrhythmic cardiac disease.

Electrocoagulation: Bipolar or heater probe coagulation is effective for treatment of angiodysplasia in the colon or upper gastrointestinal tract. The risk of perforation with heater probe coagulation may be increased in the colon and small bowel, beyond the duodenum. Monopolar coagulation may be less effective and is associated with an increased rate of complications.

Mechanical hemostasis: Mechanical hemostatic methods such as endoscopic clips have the advantage of avoiding tissue injury, which may be particularly desirable in patients taking anticoagulants and/or antiplatelet agents, or in patients with coagulation defects.

Another mechanical method that has been described in some case reports is band ligation[119], that is safe and

effective for the treatment of acutely bleeding small bowel vascular lesions with similar results to APC (recurrent bleeding in 43%) and which can be a definitive treatment for Dieulafoy's lesion.

Angiography

Angiography is indicated in patients with GI bleeding who fail to respond to medical and/or endoscopic therapy, as an alternative to surgery in hemodynamically unstable patients with severe bleeding or for patients with ongoing or recurrent bleeding following attempts to control the bleeding endoscopically. Angiographic therapies include the infusion of vasoactive drugs (vasopressin) or the delivery of agents to mechanically occlude the vascular supply of the bleeding lesion (embolization).

Vasopressin causes generalized vasoconstriction via a direct action upon vessel walls, especially the arterioles, capillaries, and venules. It should be used with caution in patients with coronary artery disease, congestive cardiomyopathy, severe hypertension, or severe peripheral vascular disease. Other side effects are arrhythmias and water retention leading to hyponatremia.

Agents used for embolization include biodegradable gelatin sponge, polyvinyl alcohol particles, liquid agents, and metallic coils. Microcoils have become the preferred agent for embolizing bleeding vessels and can be deployed by means of a microcatheter to the site of bleeding. The complications of embolization include those associated with arteriography itself (*e.g.*, hematomas, arterial thrombosis, dissection, embolism, and pseudoaneurysm formation), and bowel infarction.

The choice between vasopressin and embolization should be individualized for each patient, taking into account angiography experience. Embolization with microcoils may be more successful than vasopressin infusion (95% *vs* 80%-90%)[120,121]) but it is associated with a higher rate of complications.

Initial hemostasis may be achieved in up to 80%-95% of patients in whom angiographic therapy is technically feasible, but rebleeding is a common problem (9%-56% in embolization and 5%-50% in intra-arterial vasopressin infusion).

Surgery

Surgical therapy is reserved for patients with a known bleeding cause, found with other methods, patients with increasing transfusion requirements or life-threatening bleedings from clearly identified origins, or for cases in which haemodinamic instability does not allow the clinicians to complete the diagnostic algorithm and an IE is mandatory. In this last situation, rebleeding is usual[86,87].

CONCLUSION

Despite technological advances, OGIB is still a diagnostic challenge for gastroenterologists, with important hospital resources consumption and delayed diagnoses. WCE is the most cost-effective diagnostic procedure to identify the bleeding source and its location. In selected cases, with an outstanding severity, CT-angiography is an alternative.

Although therapy depends on the bleeding cause, BAE plays an important role in the management of lesions found in WCE. It is less aggressive than intraoperative enteroscopy and has a high index of success. A pharmacological alternative to surgery or endoscopy are depot formulations of somatostatin analogs.

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REVIEW

Role of hemostatic powders in the endoscopic management of gastrointestinal bleeding

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Abstract

Acute gastrointestinal bleeding (AGIB) is a prevalent condition with significant influence on healthcare costs. Endoscopy is essential for the management of AGIB with a pivotal role in diagnosis, risk stratification and management. Recently, hemostatic powders have been added to our endoscopic armamentarium to treat gastrointestinal (GI) bleeding. These substances are intended to control active bleeding by delivering a powdered product over the bleeding site that forms a solid matrix with a tamponade function. Local activation of platelet aggregation and coagulation cascade may be also boosted. There are currently three powders commercially available: hemostatic agent TC-325 (Hemospray®), EndoClot™ polysaccharide hemostatic system, and Ankaferd Bloodstopper®. Although the available evidence is based on short series of cases and there is no randomized controlled trial yet, these powders seem to be effective in controlling GI bleeding from a variety of origins with a very favorable side effects profile. They can be used either as a primary therapy or a second-line treatment, and they seem to be especially indicated in cases of cancer-related bleeding and lesions with difficult access. In this review, we will comment on the mechanism of action, efficacy, safety and technical challenges of the use of powders in several clinical scenarios and we will try to define the main current indications of use and propose new lines of research in this area.

Key words: Gastrointestinal hemorrhage; Endoscopy; Powders; Endoscopic hemostasis

Core tip: Hemostatic powders are a new endoscopic therapeutic modality for gastrointestinal bleeding. Based on their characteristics and mechanism of action, they may be very useful in controlling bleeding in some situations. In the last two years, a large number of studies, mainly short series of cases, have been published on this topic but their role in the management algorithm is not yet defined. In this review, we will comment on the efficacy and safety of the use of powders in several clinical scenarios and we will try to define the main current indications of use and propose new lines of research in this area.

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INTRODUCTION

Acute gastrointestinal bleeding is a prevalent condition with significant influence on healthcare costs. The annual rate of hospitalizations from acute upper GI bleeding (AUGIB) in the United States is around 160 hospital

Table 1 Hemostatic powders currently available

Name	Composition	Mechanism of action	Regulatory clearance
Hemospray™	Mineral	Absorption of water Concentration of platelets and clotting factors Mechanical tamponade	Approved in Europe and Canada[1] Under evaluation in United States
EndoClot™ PHS	Absorbable hemostatic polysaccharides	Absorption of water Concentration of platelets and clotting factors Mechanical tamponade	Approved in Turkey, Europe, Malaysia and Australia
Ankaferd® Blood Stopper	Mixture of plants	Encapsulated protein network that provides focal points for erythrocyte aggregation	Approved in Turkey

[1]For non-variceal upper gastrointestinal bleeding.

admissions per 100000 population[1], leading to approximately 300000 hospitalizations annually. Between 36% and 50% of AUGIB episodes in most published series are due to non-variceal causes, mainly peptic ulcer[2,3]. Despite improvements in medical and endoscopic therapy, mortality from AUGIB remains around 10%, with higher rates for variceal bleeding and malignancy[2]. On the other hand, severe acute lower GI bleeding (ALGIB), mainly caused by diverticular disease, vascular lesions and ischemic colitis, is an emerging cause of hospital admission[4]. In one study, the ratio of hospitalization rates between upper and lower GI complications decreased from 4.3 to 1.4 in 10 years[5].

Endoscopy plays a pivotal role in the management of both types of GI bleeding, allowing diagnosis, risk stratification and treatment[6-8]. Endoscopic hemostatic therapy is the basis of treatment in patients with active bleeding or with endoscopic features that predict an increased risk of further hemorrhage. However, endoscopic therapy in clinical practice has some drawbacks that limit its efficacy. For instance, despite being highly effective in achieving hemostasis in UAGIB, in 5%-10% of patients this bleeding will not be initially controlled or they will experience a recurrence[9]. In patients with severe acute bleeding and a difficult anatomy (*e.g.*, posterior duodenal wall or the upper region of the lesser gastric curvatures), endoscopic therapy can be challenging, often requiring a high level of technical expertise. Finally, this life-threatening condition can also present outside normal working hours when a less skilled endoscopist is on call. Therefore, a simple and effective hemostatic tool might have a significant impact on endoscopic therapy efficacy of AGIB.

Recently, hemostatic powders have been added to our endoscopic armamentarium to treat GI bleeding. They are intended to control active bleeding by delivering a substance over the bleeding site using a catheter through the working channel of the endoscope. Perhaps the main advantage of this technology is that less precision is needed, allowing for treatment of lesions with difficult access and refractory to standard therapy[10]. There are three hemostatic powders currently available for endoscopic usage (Table 1): hemostatic agent TC-325 (Hemospray™), EndoClot™ polysaccharide hemostatic system (PHS), and Ankaferd Bloodstopper® (ABS). In this review, we will describe the mechanism of action, efficacy in different clinical scenarios, safety of the hemostatic powders, and will comment on the possible role of this tool in the endoscopic treatment of GI bleeding.

MECHANISM OF ACTION

All three powders are designed to be applied through the working channel of the endoscope over the bleeding area. Their components, in contact with moisture, form a stable mechanical barrier that covers the bleeding site, inducing hemostasis. Therefore, they should only be applied if there is an active bleeding. Slight differences are found because of their different chemical composition.

Hemospray™ (TC-325)

TC-325 (Hemospray™, Cook Medical Inc, Winston-Salem, NC, United States) is a proprietary inorganic powder containing no human or animal proteins, botanicals or allergens. It is neither absorbed nor metabolized, therefore it is considered metabolically inert and nontoxic (information provided by the manufacturer). The precise mechanism of action is unknown but it is hypothesized that the powder, in contact with water, forms an adhesive covering that seals the tissue, producing a mechanical tamponade (Table 1). In 24-72 h, this adherent coat sloughs off into the lumen and is completely eliminated from the GI tract[11]. Water absorption also leads to concentration of platelets and clotting factors with activation of platelets and the coagulation cascade[12]. The *in vitro* effects of TC-325 on standardized coagulation and platelet function have been studied, showing that both prothrombin time and activated partial thromboplastin are reduced in a dose-dependent way in the presence of the powder[13]. These results suggest that Hemospray™ may facilitate local hemostasis.

EndoClot™ PHS

EndoClot™ PHS (EndoClot Plus Inc, Santa Clara, California, United States) is a starch-derived compound that consists of biocompatible absorbable hemostatic polysaccharides. In contact with blood, it rapidly absorbs water, causing a high concentration of platelets, red blood cells and coagulation proteins at the bleeding site, thus accelerating the physiological clotting cascade

(Table 1). A gelled matrix is formed that adheres to and seals the bleeding tissue. This matrix is cleared from the bleeding site in a few hours or days[14]. When applied to skin wounds, it seems to improve healing by activating fibroblasts and transforming growth factor (TGF)-β1 release[15], but this effect has not been studied in the GI mucosa.

ABS

This is a mixture of plants, including *Thymus vulgaris, Glycyrrhiza glabra, Vitis vinifera, Alpinia officinarum* and *Urtica dioica*. *In vitro* and ultrastructural studies suggest that ABS rapidly forms an encapsulated protein network that provides focal points for erythrocyte and activated leukocyte aggregation (Table 1)[16,17]. This network stems from interactions between ABS and blood proteins, such as fibrinogen, inducing protein agglutination. Total protein, albumin and globulin levels also decrease in serum, probably *via* agglutination of these molecules in the growing protein network. However, most coagulation factors are not affected by the addition of ABS to fresh normal plasma or serum[18].

ABS also has functional properties, inhibiting fibrinolysis and some natural anticoagulant pathways via interaction with the protein C anticoagulation pathway. On one hand, it enhances the expression of plasminogen activator inhibitor 1 (PAI-1), one of the major inhibitors of fibrinolysis, thus increasing clot stability. On the other hand, ABS has also been shown to down-regulate the endothelial cell protein C receptor (EPCR), a natural enhancer of protein C activity, therefore taking part in inactivation of factors Va and VIIIa[19].

EVIDENCE SUPPORTING THE ROLE OF POWDERS IN ENDOSCOPIC HEMOSTASIS

The evidence supporting the role of powders in GI bleeding is of moderate quality and based mainly on short series of cases and retrospective studies without a control group.

Hemospray™ (TC-325)

Animal models: This powder has been tested in animal models of arterial gastrointestinal bleeding. Giday *et al*[10] performed a randomized controlled trial on 10 pigs allocated to treatment with TC-325 or sham after surgical creation of an arterial bleeding from a gastroepiploic vessel opened up to the gastric lumen. The endpoint of the study was the proportion of animals in which hemostasis was achieved at 1 h. In the treatment group, acute hemostasis was achieved in the whole group with no rebleeding in the first 6 h compared to 0% of animals in the sham group. Mean time to hemostasis was 13.8 min.

Ulcer bleeding: Sung *et al*[11] carried out a pilot study on the efficacy of TC-325 as the primary hemostatic method in 20 patients with active peptic ulcer bleeding. Hemostasis was achieved in all but one (95%), a patient with a Forrest Ia ulcer who ultimately needed embolization to stop bleeding. Two patients met the criteria for rebleeding during follow-up, but no active bleeding was detected in the second-look endoscopy. However, it must be pointed out that most treated bleedings were moderate and the only patient with a high risk lesion had a worse outcome.

Cancer-related GI bleeding: Conventional therapy in this kind of bleeding has moderate success and high rates of rebleeding. Chen *et al*[20] reported on a short series of 5 patients with upper GI bleeding secondary to gastroduodenal tumors. The authors reported control of bleeding in all cases with only one case of rebleeding in a patient with disseminated intravascular coagulation. Leblanc *et al*[21] treated 5 patients with bleeding from GI neoplasms (2 esophageal, 2 gastric and 1 pancreatic) with TC-325. Successful hemostasis was achieved in all patients. Two patients showed recurrent bleeding, again successfully treated with TC-325.

Patients on antithrombotic therapy: Hostel *et al*[22] evaluated 16 patients with upper GI bleeding treated with TC-325 either as a monotherapy or as salvage therapy. In 9 patients, the source of bleeding was a peptic ulcer and in 2, a neoplasm. Eight patients were on antithrombotic therapy (ATT), including patients on antiplatelet agents, NSAIDs or VKA/heparin. Initial hemostasis was achieved in 5/8 patients on ATT and in all patients of the non-ATT group (P = 0.2). The source of bleeding was a spurting arterial vessel in two of the three failures of TC-325 in patients of the ATT group. Rates of rebleeding were similar in both groups (around 25%) and in most cases bleeding was retreated with TC-325.

Bleeding secondary to a therapeutic intervention: Leblanc *et al*[21] used TC-325 to control bleeding after a therapeutic endoscopic intervention in 13 patients (5 esophageal EMR, 4 duodenal EMR, 2 ampullary resections and 1 biliary sphincterotomy). The powder achieved complete hemostasis in all patients, either as a first-line treatment or a rescue therapy, including 2 cases with spurting arterial vessels. There were no rebleedings. Very recently, TC-325 has been successfully applied to a severe bleeding after endoscopic ultrasound-guided pseudocyst drainage which had been refractory to adrenaline and fibrin glue injection[23], and in a case of bleeding after a rectal submucosal endoscopic dissection[24].

Bleeding related to portal hypertension: TC-325 has been used in cases of both esophageal and gastric variceal bleeding with good short-term results[25-27]. Smith *et al*[28] controlled acute bleeding from severe portal hypertensive gastropathy in 3 patients. However, it is only able to control the initial bleeding and cannot prevent further bleed-

ings.

Lower GI bleeding: TC-325 has also been used for lower GI bleeding[29] which is currently not a licensed use of the powder. In the largest series published to date, 9 patients with lower GI bleeding were treated with TC-325, 4 of them with post-polypectomy bleedings. Successful initial hemostasis was achieved in all patients, with 2 cases (22%) of rebleeding[30]. Smith *et al*[28] treated one patient with a portal hypertensive colopathy with TC-325, achieving a decrease in transfusion requirements. Very recently, Kraft *et al*[31] described the use of TC-325 for the treatment of a lower GI bleeding from diffuse colonic ulcers secondary to diclofenac.

Larger case series: A multicenter European trial has been published on the use of Hemospray™ in non-variceal upper GI bleeding[32]. In this trial, 63 patients with a variety of indications, including ulcers, tumors and post-therapeutic bleeding, were treated with Hemospray™ as either monotherapy or second-line therapy. Primary hemostasis was achieved in 85% of patients when Hemospray was used as monotherapy. Seven patients rebled by the 7th day, therefore 15 patients (27%) failed to achieve sustained hemostasis. The 3 patients who rebled from a peptic ulcer had a Forrest Ia lesion. Hemospray was used as a second-line therapy in 8 patients, with two early rebleedings.

Very recently, a case series from two Swiss hospitals evaluated the performance of Hemospray™ on 16 patients with bleeding from different sources. In most cases, the powder was used as a rescue therapy with an initial hemostasis rate of 93%. Two patients rebled (12.5%), both presenting with oozing bleeding in the previous endoscopy[33].

EndoClot

There is only one publication in a peer reviewed journal reporting on EndoClot in control and prevention of EMR-related bleeding. EndoClot was applied to mucosal defects after resection of 181 lesions (82 patients) regardless of if there was immediate post-resection bleeding. Among them, 20 lesions in 18 patients had early bleeding (five of them showing spurting bleeding). Bleeding was controlled with a single round of spray in 18 lesions (90%) and two cases needed hot biopsy forceps applied to achieve hemostasis. Bleeding recurred in three of these 18 patients but no therapy was needed. The authors concluded that EndoClot effectively achieves hemostasis in controlling and preventing EMR-related bleeding[34]. Two trials on the prevention of bleeding after endoscopic mucosal resection (NCT01496781 and NCT01735786) are ongoing but there are no data available yet[35,36]. Finally, there are some studies presented only in abstract form on short series of patients with a variety of bleeding lesions, reporting a success rate of around 80%, including some with coagulation disorders[37-39].

ABS

This agent has been approved in Turkey (Table 1) for clinical hemorrhages refractory to conventional hemostatic measures. There are several reports on the mechanism of action and clinical efficacy of ABS, almost all from the same Turkish groups.

Animal studies: Several authors have shown that ABS has a clinically meaningful hemostatic effect in rats and swine models with arterial sections, skin lacerations and liver puncture wounds, even if they were treated with warfarin[40-43].

Peptic ulcer bleeding: ABS has shown efficacy in peptic ulcer bleeding in some reports with a very low number of patients, including a child and a patient with thrombocytopenia[44,45].

Cancer-related GI bleeding: Several studies have assessed ABS efficacy on malignant GI bleeding, showing a good performance[46,47]. Clinical observation suggests that the hemostatic effect of ABS on malignant bleeding persists for a long time after its delivery. Some authors have suggested that this may be due to an inhibitory effect of ABS on tumor angiogenesis. A decrease in microvessel density (MVD) in tumoral sections stained with CD34 after treatment with Ankaferd has been described[48].

Other indications: Case reports on the treatment with ABS of post-sphincterotomy bleeding[49], Mallory-Weiss syndrome[44,50] and gastric post-polypectomy bleeding[51] have been reported. Two reports on the use of ABS to control esophageal variceal bleeding have also been published[52,53].

Lower GI bleeding: ABS has been applied in patients with radiation proctitis, with transient control of bleeding, but with no effect on telangiectasias[54]. There are also anecdotal cases of ABS use on post-polypectomy bleeding[50,51] and solitary rectal ulcer[55].

Larger case series: The most recent series on the use of ABS to control upper GI bleeding included 27 patients with active non-variceal UGIB[56]. Bleeding lesions were not described. In one patient, bleeding ceased after spraying isotonic saline and in the other, 26 ABS was applied. Bleeding stopped in 19 cases (73%). In 6 of the remaining 7 patients, ABS was sprayed again plus another endoscopic hemostatic method (clip, injection, APC), achieving an adequate control in all cases. The overall rate of rebleeding was 20%. Bleeding control with ABS was more difficult in patients with a coagulopathy or who were taking AAS.

TECHNICAL ISSUES

Hemospray™

The Hemospray™ package includes a delivering device

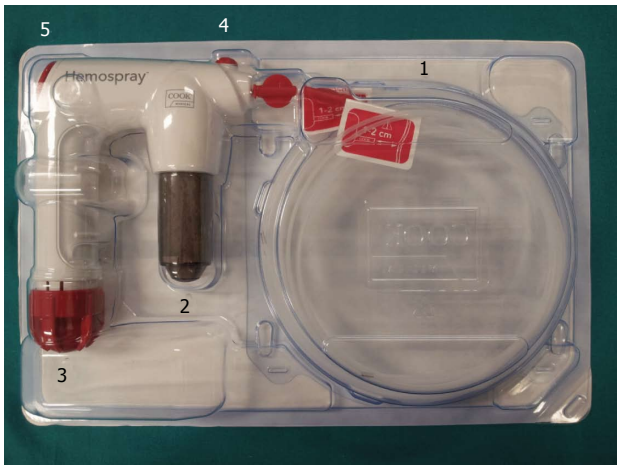

Figure 1 Hemospray™ package. 1: Spray catheters; 2: Powder cartridge; 3: Activation knob; 4: Security valve; 5: Trigger.

with a powder syringe (20 g each), two catheters (7 and 10 F, suitable for a working channel of 2.8 and 3.7 respectively) and a CO_2 cartridge (Figure 1). The latter is activated by turning a red knob placed at the base of the handle until it stops. Before inserting the catheter in the working channel of the endoscope, blood must be removed as much as possible and the bleeding site must be identified. Then, air is flushed through the accessory channel and the catheter is slowly advanced through it until the catheter tip is visualized. Care must be taken in not placing the catheter directly in contact with blood or the mucosa to avoid occlusion. It is advisable to maintain a 1-2 cm distance from the bleeding site during the procedure. Then, after turning the red valve placed at the top of the delivery device to the open position, TC-325 is ready to be delivered by depressing the red trigger button in 1-2 s pulses. Following the manufacturer´s instructions, no more than 3 devices (60 g) should be applied per patient. However, some authors have used up to 7 syringes in one patient without any secondary effect[11].

In a large trial, 7 of 63 patients (11%) treated with Hemospray suffered technical-related complications[32]. There were 3 blockages of the application catheter, 2 cases of the endoscope transiently adhering to the esophageal mucosa after use with the endoscope in retroflexion, 1 occlusion of the working channel of the endoscope and 1 malfunction of the CO_2 cartridge. In spite of this, most of the examiners felt that Hemospray was easier to use than conventional hemostatic methods[32].

Special indications suppose some technical challenges. Powder application is feasible with a duodenoscope, but caution must be taken with the use of the elevator to prevent plication of the catheter[21,57]. Hemospray cannot be used to control bleeding during EMR or ESD because it would obscure the resection field. However, Hemospray can be used at the end of the procedure if indicated.

EndoClot

The EndoClot™ PHS consists of a canister containing 1, 2 or 3 g of the powder, an air compressor that propels air down the catheter and a powder-gas mixing chamber attached to a delivery catheter that is introduced through the working channel of the endoscope[14]. After spraying, the bleeding site must be observed for 5 min. If bleeding recurs, the powder can be reapplied[34].

ABS

ABS can be delivered through the working channel of the endoscope by injecting 50-mL vials through a disposable catheter. Topical application of ABS must completely cover the bleeding surface. Following the author´s recommendations, a spray catheter or a wash pipe should be used. The amount of powder to be applied is dependent on the extent of bleeding. During administration of ABS, a local discoloration may be observed that together with the network formation may hamper the detection of the bleeding point. Therefore, the application of ABS should be performed only after precise location of source of bleeding.

SAFETY

The main theoretical concerns of using powders on an active bleeding site are local damage because of foreign body reactions and systemic embolization because of the introduction of particles into the blood stream. Embolization is of concern, especially in the case of Hemospray™ and Endoclot™ in which the powder is delivered by means of a system of positive outflow pressure. Another theoretical problem for the three powders may be bowel obstruction, caused by the formed matrix itself when it is sloughed off from the gastrointestinal mucosa a few days after its application. These secondary effects have been more extensively studied for Hemospray™, while there are very few available data about secondary effects of Endoclot and Ankaferd.

Preclinical studies

Studies with TC-325 carried out on animal models with open wounds showed endothelial and transmural damage in transected vessels in pigs, along with small clots and powder residues in lungs[58]. However, these studies referred to external wounds and more severe vessel injuries than the standard vessel defect in a GI bleeding. In the animal study by Giday *et al*[10] on gastric arterial bleeding, necropsy of the animals treated with TC-325 showed no foreign body granuloma and no signs of embolization in brain or lungs. The same group, in a study designed to identify local and systemic secondary effects following endoscopic application of TC-325, showed no local or regional particulate effects and no distance embolic effects[59]. In similar studies using Ankaferd, these secondary effects have also not been described[42]. Bowel obstruction has not been described in animals[59].

Table 2 Possible indications for the use of hemostatic powders

Primary hemostatic method	Adjuvant therapy
Lesions with a difficult endoscopic access	Failure of conventional methods
Less experienced examiner	
Malignant gastrointestinal bleeding	
Massive bleeding as a mean to achieve an initial hemostasis	

Clinical studies

There are no trials specifically designed to address secondary effects of powders. However, a recent European multicenter study has shown no secondary effects when using TC-325 for a variety of indications, including peptic ulcers, vascular lesions, malignancies and post-therapeutic bleedings[32]. No secondary effects of Ankaferd and EndoClot have been described in the scarce literature available. Bowel obstruction has not been described with TC-325, even when maximum doses were delivered[11]. Intestinal blockage seems to be rare with EndoClot because in most cases bleeding is controlled only with 3 g of powder and starch particles are rapidly degraded in the GI tract.

Regarding Hemospray™, specific concerns have been raised for some indications. For instance, when treating bleeding from esophageal or gastric varices, thromboembolism may be an issue because particles might enter the vascular system. In fact, its use in this setting is contraindicated by the manufacturer. However, the Hemospray™ outflow pressure is less than the intravariceal pressure of a bleeding varix when applied from a distance of 1-2 cm and no embolism has been shown in this indication[25,27]. *In vitro* coagulation time modifications caused by TC-325 do not seem to pose any clinical problem in cirrhotic patients[25]. A case of biliary blockage has been described when TC-325 was applied in a patient with post-sphincterotomy bleeding[57].

The application of a pressure spray on the resection area after EMR could theoretically cause a perforation. However, no perforation was detected in a small series[21]. Only one case of bowel perforation after treatment of a severe portal hypertensive gastropathy with TC-325 has been reported[28] but it was not clear if the perforation was related to the procedure. Following the manufacturer´s instructions, Hemospray™ use is contraindicated in patients with suspected GI perforation or those at high risk of perforation during the endoscopic procedure (information provided by the manufacturer). Some secondary effects of TC-325 when applied in the large bowel have been described. A case of abdominal cramps after each application of Hemospray™ on a resection area in the rectum was described. This patient did not show any long-term secondary effect[22].

CONCLUSION

Randomized controlled trials comparing powders with standard endoscopic methods are not yet available, thus the current evidence must be considered as moderate at best. The precise role of this technology in the therapeutic algorithm or GI bleeding is yet to be defined but from the present review some practical conclusions can be drawn.

Topical hemostatic powders seem to be effective to control both upper and lower gastrointestinal bleeding from a variety of sources. They can be used as a primary method or a second-line therapy and in combination with standard hemostatic methods. However, there is a substantial proportion of patients who fail to achieve primary hemostasis, mainly Forrest Ia peptic ulcer bleedings. In case of a primary failure, an adjuvant conventional endoscopic method can be applied after removing the adherent matrix with water flushing. There is some risk of rebleeding in the first week after the initial hemorrhagic episode, probably because the mineral matrix sloughs off from the mucosa after 24-72 h. A second-look endoscopy may be appropriate in this subset of patients with special risk of rebleeding.

Perhaps the most specific indication for the use of powders in GI bleeding is hemorrhage from a neoplastic lesion, which may have several bleeding points. Powders may be very useful in this setting because, when applied, they cover a large area of mucosa. Failure to achieve hemostasis with conventional methods is the other main indication for powders (Table 2).

However, active research is needed to clarify grey areas, like secondary effects and long-term efficacy. Future areas of research should be the development of well-designed randomized trials to assess efficacy of powders *vs* conventional endoscopic treatment as a primary therapeutic option, paying special attention to safety issues. Possible outcomes would be rates of rebleeding, need for adjuvant endoscopic therapy, and transfusion requirements. Sample size should be large enough to evaluate the efficacy of powders in the management of high risk bleeding lesions (*e.g.*, Forrest Ia). Rebleeding may be an underreported event in the literature; therefore, long-term efficacy must be addressed in the incoming trials. Long-term secondary effects on GI mucosa should also be addressed. Finally, since many conventional hemostatic methods are considerably cheaper, an economic analysis of the use of powders on GI bleeding should also be carried out. Larger trials, which may give response to some of these answers, are eagerly awaited.

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REVIEW

Predictors of response to anti-tumor necrosis factor therapy in ulcerative colitis

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Abstract

Ulcerative colitis (UC) is an immune-mediated, chronic inflammatory disease of the large intestine. Its course is characterized by flares of acute inflammation and periods of low-grade chronic inflammatory activity or remission. Monoclonal antibodies against tumor necrosis factor (anti-TNF) are part of the therapeutic armamentarium and are used in cases of moderate to severe UC that is refractory to conventional treatment with corticosteroids and/or immunosuppressants. Therapeutic response to these agents is not uniform and a large percentage of patients either fail to improve (primary non-response) or lose response after a period of improvement (secondary non-response/loss of response). In addition, the use of anti-TNF agents has been related to uncommon but potentially serious adverse effects that preclude their administration or lead to their discontinuation. Finally, use of these medications is associated with a considerable cost for the health system. The identification of parameters that may predict response to anti-TNF drugs in UC would help to better select for patients with a high probability to respond and minimize risk and costs for those who will not respond. Analysis of the major clinical trials and the accumulated experience with the use of anti-TNF drugs in UC has resulted to the report of such prognostic factors. Included are clinical and epidemiological characteristics, laboratory markers, endoscopic indicators and molecular (immunological/genetic) signatures. Such predictive parameters of long-term outcomes may either be present at the commencement of treatment or determined during the early period of therapy. Validation of these prognostic markers in large cohorts of patients with variable characteristics will facilitate their introduction into clinical practice and the best selection of UC patients who will benefit from anti-TNF therapy.

Key words: Ulcerative colitis; Infliximab; Adalimumab; Anti-tumor necrosis factor; Predictors of response; Personalized treatment

Core tip: The use of anti-tumor necrosis factor (TNF) monoclonal antibodies for the treatment of ulcerative colitis has been associated with high rates of primary and secondary non-response, important safety issues and considerable cost. Selection of patients with the highest probability to response to anti-TNF treatment would overcome these problems. Analysis of the pivotal trials and accumulated experience from clinical practice has led to the identification of certain prognostic factors for favorable or adverse outcomes. These include clinical and epidemiological parameters, biological markers of inflammation, endoscopic findings, molecular signatures and pharmacological factors. Incorporation of such predictors into the current therapeutic protocols may lead to the optimization of anti-TNF treatment in ulcerative colitis.

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response to anti-tumor necrosis factor therapy in ulcerative colitis. *World J Gastrointest Pathophysiol* 2014; 5(3): 293-303 Available from: URL: http://www.wjgnet.com/2150-5330/full/v5/i3/293.htm DOI: http://dx.doi.org/10.4291/wjgp.v5.i3.293

INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory disease of the colon, which affects almost 0.1% of the Western population[1]. Its natural history is dominated by chronic, relapsing intestinal inflammation, extra-intestinal involvement, and the development of long-term complications, which lead a considerable percentage of patients to colectomy.

Treatment for UC has been traditionally aimed against controlling acute and chronic inflammation[2]. Conventional therapy consists of 5-aminosalicylic acid (5-ASA) compounds, whereas more severe cases are handled with steroids during the acute phase and immunosuppressants (thiopurines) as the maintenance regimen. Despite the proven efficacy of these drugs, a significant number of patients do not accomplish durable remission and/or experience side effects. Furthermore, there has been a change in the therapeutic goals in UC in recent years. Traditionally, such goals have been considered the achievement of clinical remission and the avoidance of colectomy. Nowadays, however, it has become clear that treatment should include the complete elimination of active inflammation in the colon without long-term use of corticosteroids. In this context, mucosal healing and deep remission which both indicate the absence of endoscopically and biologically (*i.e.,* serological and/or fecal inflammatory markers) evident inflammation may be the ultimate endpoint. The accomplishment of such demanding endpoints has been linked to better long-term outcomes including colectomy and cancer prevention[3].

In recent years, treatment of UC has been revolutionized by the therapeutic application of monoclonal antibodies against tumor necrosis factor (TNF) as these agents offer effective long-term treatment for the most difficult cases.

ANTI-TNF TREATMENT IN UC

There are currently three monoclonal antibodies against human TNF that are licensed for the treatment of UC, infliximab (IFX), adalimumab (ADA), and Golimumab[4]. The data regarding Golimumab are limited. Therefore, our review will focus on IFX and ADA. IFX is a chimeric mouse/human IgG1 antibody that is administered intravenously. On the other hand, ADA is a humanized IgG1 antibody administered as subcutaneous injection. The two clinical scenarios for anti-TNF therapy in UC are: firstly, outpatient cases with moderate to severe UC who are refractory or intolerant to first-line treatment; and, secondly, patients with acute severe disease refractory to intravenous steroids[4]. In regards to the latter scenario, data exist only for IFX.

Recent clinical trials have established the efficacy of anti-TNF treatment in UC. In the two pivotal IFX trials, ACT 1/2, the primary short-term (8 wk) response of moderate to severe UC to IFX has been reported to be 65.5%/69.4% for clinical response and 33.9%/39% for remission, respectively (dose regimen 5 mg/kg at 0-2-6)[5]. Among patients who responded to the induction regimen nearly 50% maintained their response at week 30. Similarly, in the definitive clinical trial (ULTRA) for ADA, short-term response at week 8 was achieved in nearly 50% of patients, whereas long-term remission rate at week 52 was 17%[6].

Despite these encouraging results, the use of anti-TNF monoclonal antibodies is compromised in clinical practice by certain issues of efficacy and safety. Anti-TNF failure is an intriguing issue as it may be attributed to both disease characteristics and the drugs' interference with the immune system. Primary non-response is characterized by lack of response to induction therapy. The incidence ranges between 20%-40% for both anti-TNF agents. Switching to another drug is common practice, with a success rate of more than 50%[7,8]. On the other hand, loss of response is defined as the recurrence of the patient's symptoms following successful induction of remission. In the case of CD it has been estimated between 23%-46%[9], whereas no solid data exists for UC. It is believed that immunogenicity underlies secondary failure, as antibodies against anti-TNF drugs and reduced trough levels have been implicated in the majority of studies[10-12]. Optimization of treatment (dose increase and/or shortening of the administration interval) leads to recovery of response in 60%-90% of patients[10].

The use of anti-TNF has also been associated with safety concerns. Among the most fearful ones are: severe infectious including reactivation of latent tuberculosis, neurological manifestations and risk of neoplasia. In addition, infusion reactions and delayed hypersensitivity to IFX occurred in 10% and 1% of patients, respectively, in the ACT trials. The most significant side effects are probably associated with long-term administration and combination with other immunomodulatory medications. It should be noted that in the ACT and ULTRA studies there were no differences between active drug and placebo.

Taken together, it is currently evident that fine-tuning of the use of anti-TNF therapy in UC is required. The ultimate goal should be to achieve maximum efficacy with a minimum risk for side effects. When therapeutic strategies are designed the following parameters should be taken into consideration: (1) the patients who receive anti-TNF therapy are the ones with the most difficult-to-treat disease; (2) the drugs' efficiencies are far from perfect with high rates of primary and secondary failures; (3) the potential for serious side effects especially with chronic use; and (4) the high cost of these medications. One significant way to address these problems and optimize the clinical use of anti-TNF agents would be

Table 1 Prognostic indicators of response to anti-tumor necrosis factor treatment in ulcerative colitis

At initiation of treatment	During treatment
Clinical and epidemiological parameters	
Severity of the disease	Early clinical response
Younger age	
Duration of colitis < 3 yr	
Extensive colitis	
Laboratory indicators	
CRP	Low CRP at week 12
Hemoglobin	Drop of serum CRP
Serum albumin	Fecal calprotectin
Immunological and genetic markers	
p-ANCA	Gene expression profiling
Pre-treatment mucosal TNF-α expression	Percentages of regulatory T cells
Mucosal expression of IL-17 and IFN-γ	
Genetic polymorphisms	
Endoscopic findings	
	Mucosal healing
Treatment-related factors	
Pharmacological history	Number of IFX infusions
Exposure to immunosuppressants	Co-administration of immunosuppressants
Response to prior treatment with infliximab	Escalation of anti-TNF therapy
	IFX trough levels
	Antibodies against anti-TNF

CRP: C-reactive protein; p-ANCA: Perinuclear antineutrophil cytoplasmatic antibodies; TNF:Tumor necrosis factor; IL: Interleukin; INF: Interferon; IFX: Infliximab.

to carefully select patients in whom there is decreased probability for primary or secondary non-response. Such an approach will ensure that the patients who receive the medications are those who will most probably benefit. As almost ten years have passed since the initial application of anti-TNF therapies in UC, analyses of the pivotal clinical trials and accumulation of clinical experience has allowed the identification of such factors that signify a better response to these treatments (Tables 1 and 2). It is the purpose of the current review to summarize information regarding prognostic markers for response to anti-TNF monoclonal antibodies in patients with UC.

PREDICTORS OF RESPONSE

Prognostic factors at the initiation of anti-TNF treatment

Clinical and epidemiological parameters: Several studies have looked into the effect that the severity of the UC episode may have on the response to anti-TNF administration. In a study by Jürgens *et al*[13], 90 UC outpatients were treated with IFX and followed for 14 wk. Disease activity was quantified by use of the Colitis Activity Index (CAI). Nearly half of the patients achieved early remission at week 14. Overall, the mean CAI dropped from 10.4 points at baseline to 5.0 at week 14 ($P < 0.001$). The authors reported a significant positive association between UC activity and response to treatment with IFX. It should be noted, however, that only a small number of severe cases were included in this study.

In a second report, 191 UC patients who received at least one infusion of IFX between 2000 and 2009 were analyzed with the aim to identify predictors of response[14]. Mean follow-up was 18 mo. Failure outcomes included primary-non response, dose-escalation, colectomy and hospitalization, which were noted in 22%, 45%, 19% and 36% of patients, respectively. In contrast to the study by Jurgens, administration of IFX for the indication of acute severe colitis was associated with a 3-fold risk for unfavorable outcome.

Park *et al*[15] studied 89 Korean patients with moderate to severe UC who were treated with IFX. Following induction, 59 patients exhibited clinical response at week 8 (66.3%). None had a colectomy within one year, in contrast to 11/30 of those who did not respond. Predictors of primary non-response to the drug were the severity of the disease before initiation as well as prior cytomegalovirus (CMV) infection of the colon. Patients with a pre-treatment Mayo score ⩾ 11 had an increased risk of colectomy (OR = 5.05, $P = 0.007$).

Analysis of the large clinical trials ACT 1 and 2- offers additional information regarding prognostic factors for colectomy (*i.e.,* failure of IFX) in patients with moderate to severe UC[16]. As reported by Sandborn *et al*[16], 630 patients who participated in the ACT trials had a complete follow-up for colectomy. A baseline Mayo score of ⩾ 10 strongly increased the risk for colectomy (HR = 1.84, $P = 0.01$).

Prognostic indicators for response to ADA in UC have also been reported recently. A placebo controlled trial of ADA for UC patients with refractory disease who were naïve to biologics evaluated the short-term efficacy of the drug[17]. At week 8, 18.5% were in remission ($P = 0.031$ *vs* placebo). Study analysis identified a trend towards less efficacy in cases of more severe disease at baseline. Patients with Mayo score ⩾ 10, CRP ⩾ 10 mg/L and extensive disease responded less favorably

Table 2 Clinical trials that reported prognostic indicators for response to anti- tumor necrosis factor treatment in Ulcerative Colitis

Ref.	Type of study	No. of patients	Anti-TNF drug	Response endpoints	Predictor of response
Arijs *et al*[26]	Cohort		IFX	Endoscopic and histological healing	Mucosal gene expression signature
Armuzzi *et al*[31]	Retrospective	88 (78.4% IFX experienced)	ADA	Clinical remission (4-54 wk)	Short-term clinical remission Low CRP at week 12 (remission at week 54) [1]*Previous immunosupressant use (lower long-term remission rates)*
Armuzzi *et al*[27]	Prospective	126	IFX	Steroid–free clinical remission Mucosal healing Colectomy (12 mo)	Thiopurine-naïve status Combination treatment CRP drop to normal
Ben–Horin *et al*[10]	Retrospective	62 (CD/UC)	IFX	Loss or response	[1]*Low trough levels* *Anti-infliximab antibodies*
Cesarini *et al*[39]	Retrospective	41 (secondary loss of response)	IFX	Clinical remission Colectomy–free (52 wk)	Rapid clinical response to optimization
Colombel *et al*[3]	Prospective (ACT trials)	728	IFX	Clinical remission Clinical response Colectomy	Mucosal healing at week 8 (predictive of long-term outcome)
De Vos *et al*[32]	Prospective	53	IFX	Mayo clinical score Endoscopic remission	Fecal Calprotectin
Fasanmade *et al*[23]	Retrospective	728	IFX	Trough levels Clinical response	[1]*Serum albumin concentration*
Ferrante *et al*[21]	Cohort	121	IFX	Colectomy-free survival (33 mo)	Short term clinical response CRP > 5 mg/L [1]*Previous iv treatment with steroids/cyclosporin*
Ferrante *et al*[18]	Cohort	100	IFX	Early clinical response	Younger age pANCA-/ACSA+
Garcia-Bosch *et al*[28]	Retrospective	48	ADA	Clinical response (partial Mayo score) Colectomy (week 54)	Response to prior treatment with infliximab Early response to adalimumab
Gonzalez-Lama *et al*[20]	Retrospective	47	IFX	Clinical response Steroid-free remission Colectomy	[1]*Disease extent*
Gustavsson *et al*[35]	Placebo controlled trial	45	IFX	Colectomy (3 yr f-up)	Mucosal healing at 3 mo
Jakobovits *et al*[19]	Retrospective	30	IFX (not standard induction regimen 0-2-6)	Colectomy	[1]*Younger age at diagnosis*
Jürgens *et al*[13]	Retrospective	90	IFX	Clinical response Clinical remission (week 14)	CAI-disease activity ANCA seronegativity IL23R genotype
Lee *et al*[22]	Retrospective	134	IFX	Clinical response Clinical remission	Haemoglobin > 11.5 CRP > 3 Immunomodulator-naïve status Response at week 2 Mucosal healing
Kohn *et al*[36]	Open label	83 severe colitis	IFX	Colectomy/Death > 2 mo after first infusion (median f-up 23 mo)	[1]*Single infusion*
Li *et al*[34]	Prospective?	17 24	IFX	CRP Clinical response Endoscopic healing	Changes in percentages of Foxp3(+) Tregs (mucosal and systemic)
McDermott *et al*[30]	Retrospective	23 (86% infliximab experienced)	ADA	Failure (discontinuation of ADA) Colectomy (follow-up 22 mo)	[1]*Short–term failure (increased risk for colectomy)*
Olsen *et al*[24]	Retrospective	59	IFX	UCDAI	Mucosal TNF-a mRNA expression
Oussalah *et al*[14]	Retrospective	191	IFX (≥1 infusion)	Primary non-response Colectomy Infliximab optimization Hospitalization (median 18 mo)	[1]*Indication for acute severe colitis* *Hb ⩽ 9.4 g/dL* *Non-response*
Park *et al*[15]	Retrospective	89	IFX	Clinical response Clinical remission Colectomy	[1]*Mayo score ⩾ 11)* *CMV infection (within prior 3 mo)*
Reinisch *et al*[17]	Prospective (ULTRA 1)	390 (anti-TNF naïve)	ADA	Clinical remission at week 8	[1]*Mayo score ⩾ 10* *CRP = 10 mg/L*
Rismo *et al*[25]	Prospective	74	IFX	UCDAI	Mucosal gene expression signature (Th1 and Th17 related cytokines)

Rostholder *et al*[38]	Retrospective observational	56	IFX	Clinical remission	Escalation of infliximab therapy
Sandborn *et al*[16]	Prospective (ACT1&2)	630	IFX	Colectomy (54 wk)	[1]*Concomitant steroids* *CRP ⩾ 2 mg/dL* *Disease duration < 3 yr* *Mayo ⩾ 10*
Seow *et al*[40]	Cohort	115	IFX	Clinical remission Endoscopic improvement Colectomy	Trough levels
Steenholdt *et al*[41]	Retrospective	106 (CD/UC)	IFX	Loss of response	[1]*Trough levels* *Anti-infliximab antibodies*
Taxonera *et al*[29]	Retrospective	30 (IFX experienced)	ADA	Clinical response at week12 Colectomy (follow-up 48 wk)	Short–term response at week-12 (Associated with less withdrawal and colectomy rates)
Toedter *et al*[33]	Prospective (ACT-1)	48	IFX	Clinical response	Mucosal gene expression signature

[1]Italics correspond to prognostic factors for adverse outcome. IFX: Infliximab; ADA: Adalimumab; UCDAI: Ulcerative colitis disease activity index; HACA: Human anti-chimeric antibodies; CRP: C-reactive protein.

to ADA in the short-term. It should be noted, however, that these parameters did not strongly affect the result and their consideration as predictive factors must be cautious.

In all, the majority of studies appear to support the notion that severe UC demonstrates a less favorable response to treatment with anti-TNF monoclonal antibodies. From the pure clinical standpoint, the best candidate for anti-TNF administration may be an outpatient with moderate to severe UC but not severe disease requiring hospitalization, as defined by the criteria of Truelove and Witts.

In addition to disease severity, other clinical parameters may also affect the response to anti-TNF in UC. Ferrante *et al*[18] studied a cohort of 100 UC patients who were treated with IFX. More than half had extensive disease, were on immunosuppressants and received a single infusion as opposed to the standard induction scheme. Early clinical response was accomplished in 65% of patients. Younger age was associated with a higher percentage of early clinical response (responders: median age 35.7 years *vs* non-responders: 41.6, $P = 0.049$). Different results were obtained by Jakobovits *et al*[19] who reviewed the records of 30 patients with refractory UC who had received a single IFX infusion over the period 2000-2006. Half of the patients underwent colectomy over a median follow-up period of 140 d. In this cohort, younger age at diagnosis correlated with increased risk of surgery (colectomy: mean age 27.5 years *vs* non-colectomy 38.7 years, $P = 0.016$). In contrast, the indication before starting IFX was not relevant to colectomy rates. The number of patients in this study was too small for definitive conclusions to be drawn. In the analysis of the ACT trials duration of colitis ⩽ 3 years strongly increased the risk for colectomy (hazard ratio = 0.36, $P < 0.001$, respectively)[16]. Finally, disease extent may also affect response to treatment. Gonzalez-Lama *et al*[20] studied 47 UC patients who were treated with IFX and were followed for a mean duration of 8 mo. Pre-treatment predictive factors were sought: extent of the disease was the only factor that was related to higher response rates to IFX ($P = 0.02$). Extensive colitis appeared to respond less favorably in the short term in the aforementioned study of ADA as well[17].

Laboratory indicators: Among the various laboratory biomarkers of inflammation, C-reactive protein (CRP) has been the most extensively applied to clinical practice. The association between CRP and inflammatory activity in UC has not been equally strong as it is for Crohn's disease. Nevertheless, its relevance increases when cases of severe UC are studied. As these are the patients that usually require administration of anti-TNF agents, the predictive value of CRP for treatment efficacy/failure may be increased in this population. Ferrante *et al*[21] reported on a cohort of 121 UC outpatients treated with IFX and followed for a median of 33 mo. Eighty-one patients (67%) exhibited short-term response and 21 (17%) underwent colectomy. A value of pre-treatment CRP ⩾ 5 mg/L was an independent predictor for colectomy (HR = 14.5, $P = 0.006$). Similar results were presented in a study of 134 Korean patients with UC who had received at least one infusion of IFX[22]. At week 8, 87% and 45% achieved response and remission, respectively. A pre-treatment CRP ⩾ 3 mg/dL was predictive of clinical remission at week 8 (OR = 4.77, $P = 0.01$). The association between elevated CRP and less favorable response to anti-TNF was also confirmed in the analysis of the ACT trials[16]. A baseline CRP ⩾ 2 mg/L was significantly associated with increased colectomy risk (HR = 1.73, $P = 0.04$). Of note, several studies found an association between elevated CRP and colectomy[21]. Therefore, increased CRP may represent a strong marker of inflammation that requires potent treatment and will respond optimally to anti-TNF. Alternatively, CRP may be an indicator of refractory disease.

In the previous Korean study, high pre-treatment hemoglobin **w**as also a predictor of good response to IFX[22]. Baseline haemoglobin of ⩾ 11.5 g/dL was associated with higher probability for remission at week 8 (OR = 4.47, $P = 0008$). This is in accordance with the study by Oussalah[14] who reported that pre-treatment hemo-

globin ≤ 9.4 g/dL predicted primary non-response to IFX (OR = 4.35). This occurred in 22% of 191 treated patients who were included in the study. According to Truelove criteria low hemoglobin is an indicator of severe disease, which increases the risk of non-response to IFX. High pre-treatment hemoglobulin may reflect the presence of milder disease that responds better to anti-TNF treatment.

Serum albumin concentration may also have prognostic value. A study by Fasanmade *et al*[23] focused on the association between serum IFX and albumin concentration. Data from 728 patients who participated in two clinical trials were analyzed. A value of serum albumin that was outside the normal range was directly related to trough IFX levels and clinical response. Patients with low serum albumin had reduced IFX concentration and worse clinical outcomes. This correlation may reflect a common clearance pathway for albumin and anti-TNF antibodies that belong to the IgG class of immunoglobulins. In all, measurement of albumin before commencement of treatment may serve as a predictive marker of the drug's pharmacokinetics.

Immunological and genetic markers: In recent years significant advances have taken place in our understanding of the immunopathogenesis of UC. In addition, genome wide association studies have discovered polymorphisms which confer susceptibility to or protect from developing UC. These studies led to the identification of several immunological markers with may serve as indicators of disease activity and severity. The possibility that such markers may also serve as predictors of response to treatment, in particular to therapy with anti-TNF monoclonal antibodies, has been increasingly explored.

One of the classical immunological markers that are associated with UC is the presence of perinuclear antineutrophil cytoplasmatic antibodies (p-ANCA). In two recent studies absence of this marker was strongly associated with better response to IFX. In a retrospective study of 90 patients who were evaluated up to week 14 on scheduled IFX infusions, negativity for p-ANCA (along with disease severity and IL23R genotype) was predictive of IFX efficacy[13]. Similar results were obtained in the study by Ferrante *et al*[18]. The authors followed 100 UC patients treated with IFX (84 patients received a single infusion). ANCA seronegativity served as predictor of good response. Notably, a serological phenotype of ANCA+/ASCA- status was particularly correlated with lower rates of response ($P = 0.049$).

During acute flares of UC an abundance of inflammatory mediators are upregulated at the intestinal mucosa and can be detected at both the mRNA and the protein level, whereas, anti-inflammatory treatment is paralleled by a decrease or even disappearance of these markers. Therefore, such markers may hold predictive value for the response to anti-TNF treatment. A first obvious target has been TNF itself. Olsen *et al*[24] looked for predictive factors of response to induction treatment (weeks 0, 2, 6) with IFX in a cohort of 59 patients with moderate to severe disease. The outcome was assessed based on UC disease activity index (UCDAI). Among various parameters elevated pre-treatment mucosal TNF-a expression was the only independent predictive factor of clinical and endoscopic remission ($P = 0.01$ and $P = 0.003$, OR = 2.5 and 4.8, respectively).

UC-related intestinal inflammation has been characterized by upregulation of several components of the major adaptive immunity pathways (Th1, Th2, Th17). A recent study looked at the expression of the pivotal Th1 (IFN-γ) and Th17 (IL-17) cytokines before and after treatment with IFX[25]. Mucosal cytokine profile was determined by PCR and confirmed by immunohistochemistry in biopsies of 74 UC patients. Efficacy was evaluated after 3 infusions and was based on UCDAI. High pre-treatment mucosal expression of IL-17 and IFN-γ significantly correlated with remission after induction therapy (OR = 5.4, $P = 0.013$ and OR = 5.5, $P = 0.011$, respectively).

In a much broader approach, Arijs *et al*[26] performed a gene-array study in mRNA from colonic mucosal biopsies obtained from UC patients who received induction therapy with IFX. Analysis of the arrays revealed genes that were differentially expressed among responders and non-responders. Genes that showed a highly differential expression were osteoprotegerin, stanniocalcin-1, prostaglandin-endoperoxide synthase 2, interleukin 13 receptor alpha-2 and interleukin 11. The sensitivity and specificity in predicting response to IFX based on this gene profiling was 95% and 85%, respectively.

The effect of genetic polymorphisms to response to treatment remains unknown. In the aforementioned study by Jurgens the effect of UC-associated, IL-23R variants on the efficacy of IFX was reported[13]. In this study of 90 patients, homozygosity for the IBD-risk-increasing IL23R variants was associated with higher probability to respond to IFX than homozygosity for IBD-risk-decreasing IL23R variants (74.1% *vs* 34.6%; $P = 0.001$).

Treatment-related factors: Several studies have shown that the pharmacological history plays an important role in the response to anti-TNF treatment. In the study by Ferrante *et al*[21], 121 UC patients received IFX and were followed-up for a median of 33 mo. Colectomy was performed in 21 patients (21%). Previous *iv* treatment with steroids and/or cyclosporine significantly increased the risk for colectomy (HR = 2.4, $P = 0.033$). A similar association was seen in the study by Oussalah *et al*[14]. Previous use of cyclosporine was a positive predictive factor for colectomy (hazard ratio = 2.53). Finally, in the analysis of the colectomy rates in the context of the ACT-trials patients who were on steroids when IFX was started had an increased risk for surgery (HR = 1.84, $P = 0.01$)[16]. However, caution is required for the interpretation of these associations, which should take into consideration the severity of the disease. Indeed, in all of these studies

more severe disease was associated to adverse outcomes and less favorable response to anti-TNF. Therefore, the use of *iv* steroids and/or cyclosporine may simply reflect severe disease.

The association between exposure to immunosuppressants and efficacy of anti-TNF therapy merits special attention. Converging lines of evidence indicate that immunosuppressant-naïve patients respond better to anti-TNF. The efficacy of IFX was evaluated in a cohort of 126 steroid-dependent patients[27]. Approximately half of the patients achieved steroid-free remission, whereas mucosal healing at 12 mo was accomplished in one third. Thiopurine-naïve status was positively associated to steroid-free remission as well as mucosal healing at 12 mo (HR = 2.8 and OR = 3.6, respectively). In the aforementioned Korean study[22] immunomodulator-naïve status was an independent predictors for early clinical remission (OR = 4.89, $P = 0.01$). This consistent finding is in agreement with the growing evidence regarding earlier introduction of biologics in patients with moderate disease, as patients who never received thiopurines may had suffered a shorter disease course.

Finally, for patients who receive ADA as a second anti-TNF monoclonal antibody, the treatment efficacy is affected by the response to prior treatment with IFX. This was shown in a recent retrospective study that evaluated the clinical response and colectomy rate in a cohort of 48 UC patients treated with ADA[28]. The majority (81.3%) was previously exposed to IFX. Early response to ADA at week 12 was significantly more frequent in patients who achieved remission on prior treatment with IFX ($P = 0.01$).

Prognostic factors during anti-TNF treatment

Several recent studies have provided evidence to support the notion that patients with early response to anti-TNF (*i.e.,* within 3 mo) are the ones who will also benefit in the long-term. Early response was defined by a variety of clinical and biological markers in these publications.

Clinical parameters: A Spanish study evaluated the efficacy of ADA in 48 UC patients who were followed-up to week 54[28]. In this cohort the only predictive factor for colectomy was the absence of early clinical response, which was determined by partial Mayo score at week 12 (colectomy: 14.7% *vs* no colectomy: 42.9%, $P = 0.035$).

These results were replicated in a cohort of 121 UC outpatients[21] Eighty-one patients initially responded to IFX with 2/3 maintaining clinical response throughout follow-up. Twenty-one patients ended up with colectomy after a median follow-up of 33 mo. No predictors for durable response were identified. Colectomy on the other hand strongly correlated with early non-response to IFX (HR = 10.8, $P < 0.001$).

In the study by Lee *et al*[22], 45% of 134 patients with UC who received at least a single IFX infusion, achieved remission at week 8. Short-term remission rates were higher in patients who responded very early, at week 2 (OR = 20.54, $P = 0.006$).

The value of ADA in 30 UC patients who had failed IFX was studied retrospectively[29]. Response and remission rates were assessed at weeks 4 and 12 and colectomy rates over a mean follow-up of 48 mo. In the long-term 50% were still on ADA and 20% underwent colectomy. The risk of surgery was higher for patients who did not achieve response at week 12 ($P = 0.001$).

Similarly, Mc Dermott *et al*[30] studied 23 patients who received ADA induction and maintenance treatment. Of note, 86% had previously failed IFX. Discontinuation of ADA over a follow-up period of 22 mo was the primary endpoint and occurred in 70% of patients. Colectomy-free survival at 24 mo was 59%. The only factor associated with increased risk for surgery was the absence of early response to ADA. Among patients who underwent colectomy, 55% had failed ADA at week 12.

Armuzzi *et al*[31] evaluated the short- and long-term effects of ADA in 88 UC patients out of whom 78% had previously received IFX. The rates of clinical remission increased from 17% to 43% at weeks 4 and 54, respectively. Interestingly, achievement of early remission as well as low CRP at week 12 predicted remission at week 54 (OR = 4.17 and 2.63, respectively).

Laboratory indicators: The same conclusion regarding the predictive value of early response was obtained when laboratory markers of inflammation were studied. We already mentioned the predictive value of low CRP at week 12 in the study by Arnuzzi[31]. In another publication from the same group regarding 126 steroid-dependent patients who received IFX[27] drop of serum CRP value to normal after the induction-regimen predicted steroid-free remission and mucosal healing at 12 mo (HR = 4.6, OR = 6.0, respectively). Similar results were reported in a study that used fecal calprotectin as an inflammatory marker. Serial weekly measurements of fecal calprotectin were performed in a cohort of 53 patients who received IFX[32]. Two thirds of patients achieved endoscopic remission at week 10, whereas the median calprotectin level significantly drop from baseline ($P < 0.001$). Early reduction of calprotectin at week 2 predicted endoscopic remission. At week 10, clinical and endoscopic remission strongly correlated to fecal calprotectin concentration.

Immunological markers: Early post-IFX changes of the mucosal and peripheral immunophenotype of UC patients showed strong correlation with clinical response to the drug. Toedter *et al*[33] studied 113 colonic biopsies from 48 patients who participated in the ACT-1 trial. Biopsies were taken before and after treatment with IFX up to week 30. Gene expression profiling was performed. The investigators were able to identify certain genes that demonstrated significant alterations in patients that responded to treatment with IFX but not in non-responders.

In a study that included both Crohn's and UC, the

effect of IFX on the percentages of regulatory T cells (Treg) was investigated[34]. Flow cytometry, PCR and immunohistochemistry were applied to quantify the expression of Forkhead box protein3 (Foxp3)-positive T cells in both peripheral blood samples and mucosal biopsies before and after IFX treatment. Responders to IFX were characterized by significantly increased numbers of CD4(+) CD25(+) Foxp3(+)Treg and CD4(+) CD25(-) Foxp3(+) Tregs in blood ($P < 0.05$) and a significant down-regulation in the tissue ($P < 0.001$). The duration of clinical response to IFX correlated to a sustainable peripheral increase of Foxp3 (+) Treg cells.

Although such individual molecular characterization is far from being clinically applicable, it shows that personalized therapy which will be based on the particular immunophenotype may guide the therapeutic approach in the future.

Endoscopic findings: In recent years, mucosal healing (*i.e.,* the disappearance of visible active inflammatory lesions in endoscopy) has emerged as a definitive endpoint in the natural history of UC and an indispensable therapeutic target both in clinical trials and "real-life" practice. This is because mucosal healing has been shown to be associated with sustained long-term remission in patients with UC[3].

In the pivotal ACT trials endoscopic evaluations were performed at various time points and mucosal healing was defined as Mayo subscore of 0 (normal) or 1 (mild). Early endoscopic improvement at week 8 was associated to improved clinical outcomes[3]. Accordingly, low endoscopy subscores at week 8 predicted reduced risk of colectomy through week 54 ($P = 0.0004$) as well as higher remission and steroid-free remission rates ($P < 0.0001$).

A single IFX infusion or placebo was administered to 45 patients with acute, steroid-refractory UC[35]. Three years later the beneficial effect of the drug persisted as less patients in the IFX group underwent operation (50% *vs* 76%, $P = 0.012$). Endoscopic remission at month 3 strongly predicted a reduced long-term risk for colectomy ($P = 0.02$).

Mucosal healing was also a positive predictive factor for long-term remission in the study by Lee *et al*[22]. A variety of predictors for short-term outcome were identified whereas the only parameter associated with sustained long-term benefit was endoscopic remission (OR = 4.66, $P = 0.04$).

Treatment-related factors: The number of IFX infusions was associated with improved sustained response to anti-TNF treatment. Kohn *et al*[36] studied the effect of IFX treatment in 83 patients with severe steroid-refractory UC.Patients received ⩾ 1 infusions and were followed for a median of 23 mo. Twelve out of 83 patients (15%) had a colectomy within 2 mo. The risk for a prime adverse event was significantly higher among patients who received a single IFX infusion as opposed to those who were given two or more doses (OR = 9.53, $P = 0.001$).

The combined administration with immunosuppressants appears to have an advantage in comparison to single IFX therapy. This was shown in the study by Armuzzi *et al*[27]. In this cohort of 126 steroid-dependent UC patients combination treatment with IFX and thiopurines was a predictor of steroid-free remission (HR = 2.2). In another prospective trial Panaccione studied 231 patients with moderate disease who were biologics-naïve and had not received azathioprine over the 3 mo before enrollment. Patients were offered IFX monotherapy, azathioprine monotherapy or combination treatment. Steroid-free remission at week 16 was significantly more common in the combination arm of the study ($P < 0.05$ compared to both monotherapies)[37].

The need for escalation of anti-TNF therapy is also a poor prognostic factor for long-term outcome. In a cohort of 56 patients with moderate colitis who were treated with IFX, 89% proceeded to maintenance treatment[38]. During a mean follow-up of 38 mo, clinical remission was achieved in 36% of patients at 12 mo, whereas 54% required escalation of treatment. Intensification of IFX treatment was a negative predictive factor of remission at 12 mo ($P = 0.01$). In accordance, colectomy was performed more often in the "escalation" group (33% *vs* 21%).

In a related study, Cesarini *et al*[39] showed that rapid response to escalation treatment has a favorable effect on long-term outcome. They studied the records of 41 UC patients with loss of response to IFX who were treated with either dose doubling or interval shortening. The primary outcome was rapid response which was evaluated at the follow-up visit after treatment escalation. Remission and colectomy were evaluated by week 52. The majority (90%) responded rapidly and 46% achieved rapid remission. Only 4 patients (9.8%) underwent colectomy by week 52. The main predictor for avoidance of colectomy was initial response to intensification treatment ($P = 0.002$).

Recent developments emphasize the importance of serum trough levels of IFX and ADA and the formation of antibodies against anti-TNF monoclonal antibodies for the pharmacokinetics as well as the therapeutic efficacy of these drugs. In a study of 115 UC patients on maintenance treatment, clinical outcomes were associated to IFX trough levels[40]. Detectable drug in serum predicted clinical remission and endoscopic improvement at week 54 ($P < 0.001$ for both parameters). Reduced trough levels correlated with increased risk of colectomy in this cohort ($P < 0.001$). Interestingly, antibody-status was not predictive of response to IFX treatment.

Steenholdt *et al*[41] retrospectively studied 106 IBD patients on IFX, who either maintained or lost their response. Significantly higher IFX levels and lower antibodies titer were measured in patients with sustained response to IFX ($P < 0.0001$). Moreover, the authors suggested threshold values for the two parameters to ac-

curately predict and/or explain loss of response to IFX.

Similarly, Ben-Horin *et al*[10] tested the samples of 62 mixed IBD patients for anti- IFX antibodies and serum trough levels. Low trough levels and high antibodies titer were found in 83% of patients with loss of response and in 8% of patients who maintained remission ($P < 0.001$).

Critique of available markers

As the number of UC patients who have been exposed to anti-TNF monoclonal antibodies steadily increases, more factors will be reported that may be associated with better or worse response to these medications. Before, however, their use is recommended for the selection of patients in clinical practice, careful analysis of the specifics of each marker should be performed and inherent problems with the interpretation of the results from clinical trials should be kept in mind.

Clinical markers have the advantage to be readily available and identifiable in a straightforward fashion. They are easy to use, replicable, non-invasive and, overall, convenient for use in clinical practice. Caution, however, is needed when data from clinical trials are analyzed as the definition of a certain parameter may vary between different studies. In particular, clinical response and remission may be related to a variety of activity scoring systems or arbitrarily defined clinical criteria. In addition, the time point in which a certain clinical marker is reported is of pivotal significance. This is so because UC is a lifelong condition and, therefore, only time points with significant length are relevant to a true remission. Criticism also occurs regarding RCTs in the means that they may not always include patients that reflect 'real-life' IBD populations[42].

Endoscopic markers such as mucosal healing are of significance as recent studies have shown that they are indeed associated with better disease outcomes. It should be noted, however, that the major clinical trials have defined mucosal healing as Endoscopy Mayo score of 0 or 1. Whether the latter score truly represents absolute and complete elimination of inflammation is questionable. In addition, such markers require the performance of an invasive procedure (colonoscopy) soon after the commencement of treatment ($\leqslant$ 3 mo), which may not be easily acceptable from a patient, in particular when clinical remission has taken place.

Serological markers such as CRP are also easy to obtain. Nevertheless, there has not been good correlation between CRP and clinical activity of UC with the exception of severe cases. In addition, its prognostic value has only been reported in a minority of trials, given the fact that CRP is usually determined in every case of UC. Fecal calprotectin is a good indicator of ongoing acute (neutrophilic) inflammation in the colon. However, no studies have indicated that the magnitude of pre-treatment fecal calprotectin predicts the response to anti-TNF. In addition, the measurement of fecal calprotectin is not widely applied in practice and technical issues exist regarding the standardization of methodology. It should be noted, however, that both serum CRP and fecal calprotectin may be more useful when their short-term change in response to anti-TNF is considered rather that their absolute pre-treatment values.

Immunological and genetic markers are important as they hold promise for individualized therapy based on the specific characteristics of each individual patient. The major drawbacks for the application of such markers are technical challenges and lack of replication for most results. An additional problem is the redundancy of the immunological pathways that underlie inflammation in UC. Therefore, a single marker may not be sufficient enough to cover the whole mechanism of injury. Similarly, UC is a polygenetic trait and single gene polymorphisms do not usually lead to the manifestation of the disease phenotype. Nonetheless, as additional biological drugs will become available for the treatment of UC, selection of patients according to the predominant immunogenetic pathway may become the most cost-effective approach.

CONCLUSION

Currently, no single marker fulfils all criteria for being an appropriate prognostic indicator for response to anti-TNF treatment in UC. The ideal predictor should be clearly defined, simple and easy to obtain, as well as of repetitive association between different trials. Alternatively, a predictive model which includes clinical, laboratory and even genetic and/or immunological parameters may be more difficult to develop but more accurate in its predictive value. In that context, and whilst our experience with anti-TNF therapy in UC expands, it is important to continue the search for optimal predictive factors of response or failure. Each of the proposed prognostic parameters should be validated in large populations of patients and across clinical trials of different ethnicities. Eventually, personalized treatment may be the best, safest and most cost-effective strategy in diseases with such a complex pathogenetic background.

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REVIEW

Genetic update on inflammatory factors in ulcerative colitis: Review of the current literature

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Abstract

Ulcerative colitis (UC) is one of the main types of inflammatory bowel disease, which is caused by dysregulated immune responses in genetically predisposed individuals. Several genetic factors, including interleukin and interleukin receptor gene polymorphisms and other inflammation-related genes play central role in mediating and modulating the inflammation in the human body, thereby these can be the main cause of development of the disease. It is clear these data are very important for understanding the base of the disease, especially in terms of clinical utility and validity, but summarized literature is exiguous for challenge health specialist that can used in the clinical practice nowadays. This review summarizes the current literature on inflammation-related genetic polymorphisms which are associated with UC. We performed an electronic search of Pubmed Database among publications of the last 10 years, using the following medical subject heading terms: UC, ulcerative colitis, inflammation, genes, polymorphisms, and susceptibility.

Key words: Ulcerative colitis; Inflammatory factors; Genes; Polymorphisms; Susceptibility

Core tip: Ulcerative colitis (UC) is a disorder of the idiopathic and chronic inflammation of the colonic mucosa. Several genetics factors influence the development of the disease, especially interleukin and interleukin receptor gene polymorphisms and other inflammation-related genes. In this review we collected the current literature on PubMed Database about those genetic markers that are associated with UC, we focused on the following terminology: UC, inflammation, genes, polymorphisms, susceptibility.

Sarlos P, Kovesdi E, Magyari L, Banfai Z, Szabo A, Javorhazy A, Melegh B. Genetic update on inflammatory factors in ulcerative colitis: Review of the current literature. *World J Gastrointest Pathophysiol* 2014; 5(3): 304-321 Available from: URL: http://www.wjgnet.com/2150-5330/full/v5/i3/304.htm DOI: http://dx.doi.org/10.4291/wjgp.v5.i3.304

INTRODUCTION

Ulcerative colitis (UC; MIM 191390) and Crohn's disease (CD; MIM 26600) are the two main, related forms of inflammatory bowel disease (IBD) which are chronic, relapsing inflammatory disorders of the gastrointestinal tract[1]. The highest annual incidence rate of UC was re-

ported in Europe (24.3/100000) and in North America (19.2/100000). However, in Asia and in the Middle East the rate is much lower (6.3/100000) believed to be associated with the different level of industrialization[2]. UC has a bimodal pattern of incidence, with the mean age diagnosis between ages 15 and 30 years, and a second smaller peak between ages 50 and 70 years[3]. Clinically, UC is characterized by superficial, continuous mucosal inflammation and ulcers restricted to the colon, whereas CD is a segmental, transmural disorder involving any part of the gastrointestinal tract[4].

Although the precise etiology of IBD still remains obscure, the accepted hypothesis is that in genetically susceptible individuals the commensal luminal flora trigger an inappropriate, overactive mucosal immune response causing intestinal tissue damage that is further modified by specific environmental factors (*e.g.,* smoking)[5].

At first, observational family studies and twin studies directed the interest to genetic components in the pathogenesis of IBD[6, 7]. Recently, genome-wide association studies (GWAS) have resulted in the identification of many novel single nucleotide polymorphisms (SNPs) for CD initially and latterly for UC which is thought to be more genetically heterogeneous than CD. To date, the number of known risk loci has expanded to 163, of which 110 confer common susceptibility to IBD, whereas 30 seem to be specific to CD and 23 to UC[8].

Immunologically, CD is associated with a T helper type 1 (Th1)[9] and T helper type 17 (Th17)[10] immune response, thus interferon gamma/interleukin-12 (IFNγ/IL-12) and interleukin-23/interleukin-17 (IL-23/IL-17) cytokines assign the downstream release of complex network of further pro-inflammatory cytokines (*e.g.,* IL-18, IL-2, IL-1, IL-21, IL-22, IL-17A, IL-17F, IL-26). However, UC is thought to be the result of a Th17 (IL-17) and a modified Th2 response (IL-13, IL-5 and IL-9). In addition, interleukin-6 (IL-6) and tumor necrosis factor alpha (TNFα) are produced by both T helper type 2 (Th2) cells and Th1 cells.

The IBD-associated loci encode for genes involved in maintenance of epithelial barrier integrity, antigen pattern recognition, autophagy, innate immunological response, coordination of adaptive immune responses and leukocyte recruitment (Figure 1).

Most of the difference at molecular level between UC and CD is found in human leukocyte antigen (*HLA)* Class Ⅱ genes and in genes related to pattern recognition [*e.g.,* nucleotide-binding oligomerization domains *(NODs), toll-like receptors (TLRs)*], innate immunity (*e.g., IL-23R*) or autophagy pathways (*e.g., ATG16L1, IRGM*). The *HLA* class Ⅱ genes *DR2*, *DR9*, and *DRB1*0103* were identified as susceptibility genes for UC, whereas *DR4* was a protective gene[11,12]. *HLA* haplotype *DRB1*0103* is significantly associated with disease susceptibility, extensive disease, and an increased risk of colectomy[13]. While several genes involved in bacterial sensing [nucleotide-binding oligomerization domain 2/caspase activation recruitment domain 15 (*NOD2/CARD15*)] and processing mechanisms (autophagy related genes *ATG16L1* and *IRGM*) are defective only in CD, the Th17/IL-23 axis related cytokines [*e.g.,* IL-23R, IL-12B and their downstream components signal transducer and activator of transcription 3 (STAT3), janus kinase 2 (JAK2)] have been associated with both CD and UC.

Dysfunction of the barrier integrity, enhanced permeability is also a main feature in UC. Recently, in a large review epithelial barrier genes were discussed in detail, namely, extracellular matrix protein 1 (*ECM1*), cadherin type 1 (*CDH1*), hepatocyte nuclear factor 4, alpha (*HNF4α*), and laminin beta 1 (*LAMB1*).

These genes were found not to be associated with CD, implying they may confer susceptibility specifically to UC[14]. Interestingly, the *CDH1* locus represents the first genetic association also identified in a GWAS for colorectal cancer susceptibility[15, 16].

In our review we focus on inflammation-related genes and polymorphisms including interleukin and interleukin receptor gene polymorphisms which are involved in the pathogenesis of UC.

INFLAMMATION-RELATED GENETIC FACTORS

Cytotoxic T-lymphocyte antigen 4

Cytotoxic T-lymphocyte antigen 4 (CTLA4) is an inhibitory receptor expressed by activated T cells. It is an important downregulator of the T cell activation and might contribute to peripheral tolerance. CTLA4 is a good candidate gene for susceptibility to UC, because it acts as a negative regulator of T cell activation and T/B, T/monocyte-macrophage cognate interaction. The localization of *CTLA4* gene is on chromosome 2q33. Several genetic polymorphisms have been reported in the human *CTLA4* gene[17, 18].

In a Tunisian population study, where A+49G was analyzed comparing the UC patients with the control subjects, the frequencies of the +49A allele and the homozygous +49 A/A genotype were higher in UC patients than in controls, but those differences were not statistically significant[17].

In a Dutch Caucasian and in a Han Chinese UC cohort studies the C-318T and A+49G polymorphisms of *CTLA4* gene were examined. No significant differences were observed in distribution of allele, genotype and haplotype frequencies between UC and control group[19].

A Hungarian cohort was examined for the same polymorphisms and no association was found between heterozygous AG genotype, homozygous GG variant, and G allele frequency of the *CTLA4* gene A+49G polymorphisms comparing the UC (IBD) group to the healthy controls. The A+49G does not represent an obligatory susceptibility factor for UC[20].

The A-1661G and the T-1722C two other SNPs in the non-exonic region were investigated in the Han

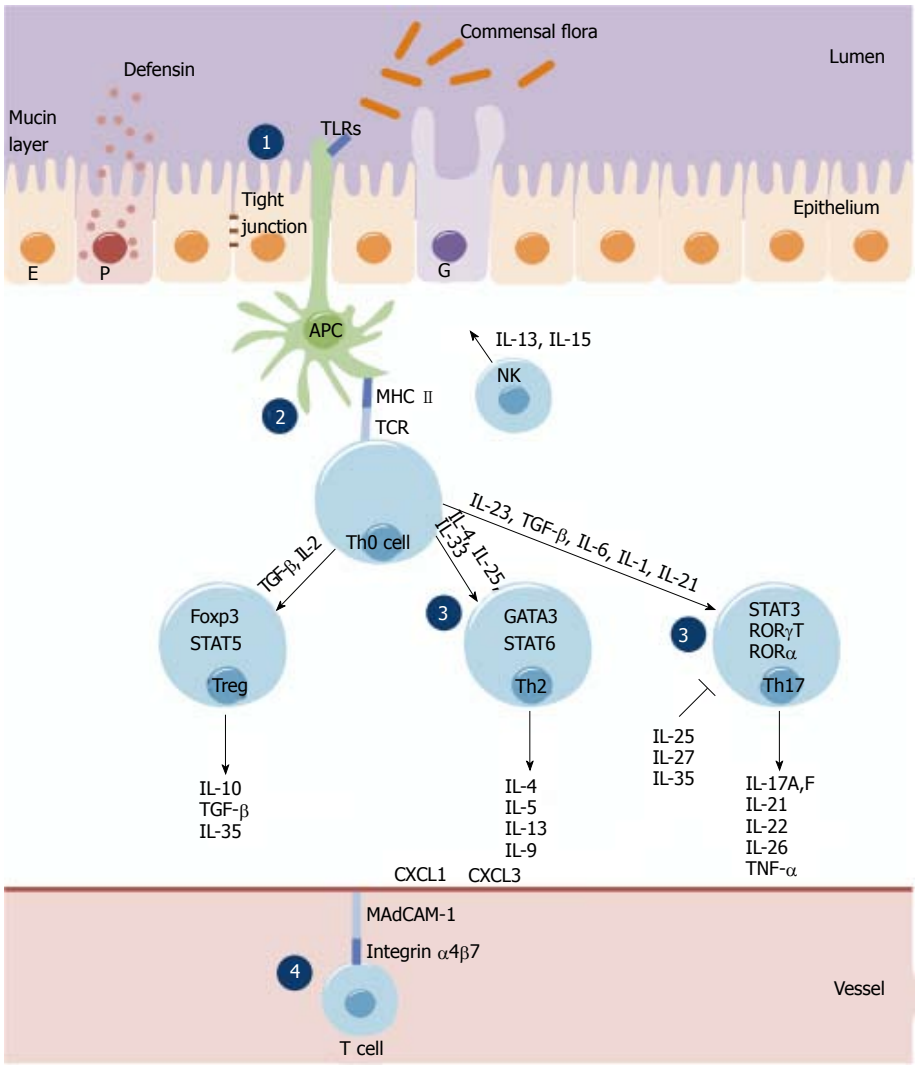

Figure 1 The Ulcerative colitis-associated risk loci. Damage of the epithelial barrier (E), the mucinous biofilm layer secreted from goblet cells (G), the antibacterial peptides (*e.g.*, defensines) produced by Paneth cells (P) and the tight junctions leads to increased permeability in ulcerative colitis (UC). Antigen presenting cells (APCs) (*i.e.*, macrophages and dendritic cells) in the lamina propria are increased in absolute number in UC, they bind microbial products through detection molecules of the innate immune system, including Toll like receptors (TLRs) on the cell surface and on the cytoplasmatic NOD-like receptors (NLRs). Stimulation of these receptors induces intracellular signaling cascades, resulting in secretion of large number of cytokines, chemokines, and immunomodulatory factors. The development of the Th2, Th17 and Treg subsets from naïve, Th0 cells during primary immune response is mainly determined by cytokines and chemokines, and is under the control of certain transcription factors: T-bet (T-box expressed in T cells), GATA3 (GATA binding protein), RORγt (retinoid-related orphan receptor γt), RORα, STATs (signal transducer and activator of transcription) and FoxP3 (forkhead box P3). Leukocyte migration and recruitment from vessels is mediated by selectins, integrins, ICAMs and chemokines (*i.e.*, c-c motif chemokine ligand, CXCL). The UC-associated loci encode genes involved in: (1) maintenance of epithelial barrier integrity (*e.g.*, ECM1, CDH1, HNF4A, LAMB1, PTGER4, SLC22A4/SLC22A5, MYO9B, MDR1); (2) antigen pattern recognition (*e.g.*, NLRs, TLRs); (3) innate and adaptive immunological responses (*e.g.*, IL-23R, IL-12B, TNFα, IL10R, JAK2, STAT3, HLA-region); and (4) leukocyte recruitment (integrin α4β7, ICAM-1, MAdCAM-1, CXCLs, CCRs).

Chinese population. The frequency of A/G + G/G genotype at position -1661 was statistically higher in UC patients than in healthy controls. The G allele frequency was also significantly increased in UC patients than in the controls. The A-1661G polymorphism of the *CTLA4* is a risk factor for UC in Han Chinese of central China. They found no association between T-1722C polymorphism and UC[18].

Janus kinase 2

Janus kinase 2 (JAK2) is a member of a family of tyrosine kinases involved in a specific subset of cytokine receptor signaling pathways. JAK2 has been found to be constitutively associated with the prolactin receptor and required for responses to gamma interferon[21-23].

In GWAS studies several UC loci were identified. The rs10758669 and the rs10974944 SNPs in the *JAK2* locus were found to be strongly associated with UC in the population from the United Kingdom[14] and Netherlands[24].

In a Korean population two SNPs (rs10758669 and rs10975003) were investigated. The rs10758669 showed no significant differences in genotype and allele distribution between UC patients and controls, while it was significant on level of genotype and allele frequencies in case of rs10975003. The rs10975003 SNP plays role in

the pathogenesis of UC in Koreans[25].

Signal transducer and activator of transcription 3

This protein is a member of the signal transducer and activator of transcription (STAT) protein family. It is encoded by the signal transducer and activator of transcription 3 (*STAT3*) gene. In response to cytokines and growth factors, STAT family members are phosphorylated by the receptor associated kinases, and then form homo- or heterodimers that translocate to the cell nucleus where they act as transcription activators. This protein is activated by phosphorylation in response to various cytokines and growth factors including interferons (IFNs), epidermal growth factor (EGF), interleukin-5 (IL-5), IL-6, hepatocyte growth factor (HGF), leukemia inhibitory factor (LIF) and bone morphogenetic protein 2 (BMP2). STAT3 relays the expression of a variety of genes in response to cell stimuli, and thus plays pivotal role in several cellular processes, such as cell growth and apoptosis[26-28].

In a large GWAS study the rs12948909 SNPs in the *STAT3* locus was identified and found to be strongly associated with UC in the United Kingdom population[14].

In the Hungarian population the *STAT3* rs744166 was investigated. *STAT3* rs744166 TT genotype and T allele frequencies were significantly higher in patients with UC than in controls. Logistic regression analysis revealed that the TT genotype confers as an increased risk for the development of UC[23].

In a North American study the same polymorphisms were tested, but no significant differences were found between the UC group and healthy controls[29].

Tumor necrosis factor alpha

The pro-inflammatory cytokine, tumor necrosis factor alpha (TNFα) has important pathogenic role both in CD and in UC[30,31]. Through its ability to cause epithelial barrier disruption in colonic epithelial cells[32,33] it is responsible for tissue damage. The *TNFα* gene can be found at the inflammatory bowel disease 3 *(IBD3)* locus within the major histocompatibility complex (MHC) region. In several studies it has been found as a susceptibility locus for IBD[34-36]. Level of TNFα is elevated in serum, stools, and inflamed bowel mucosa of patients with IBD[37-41].

The polymorphism at position -308 is a point mutation, where the presence of G defines the common variant *TNF1*, and A the less common variant *TNF2*. Susceptibility to UC has been positively[42] and negatively[43] associated with carriage of *TNF2* allele. Some studies suggested that this allele might have a small but significant association with greater levels of TNF transcription[44, 45]. However other authors did not find any influence of *TNFα* bi-allelic polymorphism on UC susceptibility, although they reported a higher frequency of the *TNF2* allele in women with extensive disease compared with those with distal colitis[46]. In Mexican Mestizo UC patients increased frequency of *TNF2* allele and *TNF* 1/2 genotype was found, suggesting this could be an additional genetic marker for the susceptibility to UC[47]. Similar findings have been reported in patients with UC with Caucasian origin[42, 46, 48, 49].

The *TNFα* polymorphisms A-308G and T-857C increase the TNFα production, raising the possibility of correlation with different disease course or response to therapy[50]. The A-238G and A-308G in *TNFα* promoter region have been found as a susceptibility factor in different autoimmune disorders, including asthma[51,52], psoriasis[53] and rheumatoid arthritis[54]. The polymorphism A-238G was associated with lower production of TNFα in Caucasian UC patients[46].

In a New Zealand Caucasian UC cohort was found, that carriers of the *TNFα* -308A allele may give higher risk for pancolitis and the necessity for bowel resection[55]. In Israeli Jewish patients having CD or UC, the allele and carrier frequencies of -857T allele did not differ between IBD patients and controls, suggesting this SNP in Ashkenazi Jewish patients neither determines the susceptibility, nor influences the clinical phenotype of CD or UC[56].

Different studies supported that *TNFa* -308 in UC may be an ethnic population-specific risk factor. Studies from East Asia suggested strong association of the *TNFα* -308 gene promoter polymorphism for UC in East Asians. Allele frequency of *TNFα* -308A was significantly higher in Han Chinese UC patients than in healthy controls. Haplotype analysis revealed 6 haplotypes including H5 (*TNF* 1031T/863C/857C/380G/308A/238G/) and H3 (*TNF* 1031C/863C/857C/380G/308A/238G/). Haplotype frequency of H5 was significantly higher in UC patients, suggesting that H5 is associated with UC and the *TNFα* gene may be a susceptibility gene to UC[57]. Interestingly, the meta-analysis did not reveal any association of the *TNFα* -308 gene promoter polymorphism with UC in Europeans[58]. In a Caucasoid population from the North of Spain the *TNFα*-308 alleles did not influence the appearance of steroid dependency either in UC or in CD[59]. In Italian pediatric patients the *TNFα* -308 was significantly increased in patients with UC[60].

In Czech pediatric IBD patients significant differences in TNFα -308 A polymorphism were found between UC group and controls, but no differences were noted between this polymorphism and the clinical characteristics of UC[61].

Significant correlation of the *TNFα* -863A variant was demonstrated with colonic disease and greater height at diagnosis[62], but in this study they could not find any significant difference for the -857 allele. In patients with UC only a trend toward an increased frequency of steroid resistance was found in carriers of the *TNFα* risk genotype compared to non-carriers[60].

In the Han Chinese population the *TNFα* C-1031T, A-863C and T-857C allele/carrier frequencies were analyzed between UC patients and healthy controls. They did not find any significant difference of the tested al-

lele/carrier frequencies between UC patients and controls, only the *TNFα* -857T was increased in UC patients but did not reach statistical significance[57].

Organic cation transporter 1/2

OCTN1 *(SLC22A4)* and OCTN2 (*SLC22A5)* are widely expressed [63-66], but specifically expressed in principal intestinal cell types affected by CD: epithelial cells, CD68+ macrophages and CD43+ T cells. *SLC22A4* and *SLC22A5* encode the polytopic transmembrane sodium-dependent carnitine and sodium-independent organic cation transporters OCTN1 and OCTN2[67]. OCTNs have important role in the maintenance of intracellular homeostasis and in the energy production of the cell [68]. Both OCTNs play important role in the maintenance of gastrointestinal health and in the prevention of gut inflammation[69, 70].

CD associated variants, the *OCTN1* T1672C and *OCTN2* -207G were as strongly associated with UC in unrelated Caucasian subjects[71].

Homozygous patients for the *OCTN1* 1672T variant were significantly associated with UC in a study cohort from Italy, suggesting that OCTN1 could have a role in modulating the severity of chronic inflammation in UC[72].

The mutation that leads to L503F substitution in the OCTN1 protein can alter the transporter's activity[73, 74]. Only a weak gender-specific effect of L503F was observed at male UC patients in a cohort of familial and sporadic IBD from the central Pennsylvania, United States[75].

Multidrug-resistant transporter-1

The multidrug-resistant transporter-1 (*MDR1*) gene encodes the transmembrane protein, P-glycoprotein 170 (Pgp)[76]. This gene is an excellent candidate gene for the pathogenesis of IBD[77]. Pgp functions as an ATP-dependent efflux transporter pump and is expressed in many normal tissues like in the epithelial surfaces of the intestine, biliary ductules, proximal tubules of kidneys and central nervous system[78-80].

One of the most significant *MDR1* gene mutations is the C3435T polymorphism. Decreased expression of the *MDR1* gene and lower Pgp activity has been associated with this variant. However, studies showed conflicting results. In a German study the T allele and TT genotype frequencies of C3435T polymorphism were significantly increased in UC patients[81]. Glas *et al*[82] found in a small group of UC patients partial accordance with a trend towards an increased frequency of T allele compared to controls, but a statistical difference was detected only in one of two different control groups. In a meta-analysis significant association of the 3435T allele and the 3435TT genotype has been found with UC[83]. The triallelic G2677T/A and the C3435T have been shown to correlate with Pgp expression[84-87]. Significant association of C3435T and G2677T was detected with UC: UC patients had significantly higher frequency of 2677T allele and of the 3435TT genotype. Haplotype analysis revealed that carriers of 3435T/2677T haplotype have significantly higher risk of having UC[88]. In a Japanese UC cohort the C3435T was predictive of susceptibility to later onset UC, but not for the early onset of UC[89].

Large study with German and British UC and CD patients failed to demonstrate association. It was confirmed, that this SNP is associated with UC especially in patients with extensive colitis[90]. In addition completely negative findings have been reported in large studies from North America[7], Slovenia[91] and Italy[92]. Similarly to these results, UC patients with Caucasian origin from central Poland were found that *MDR1* C3435T polymorphism is not a risk factor for IBD, including both UC and CD[93].

A study with New Zealand IBD patients supported the role of *MDR1* as a candidate gene for UC. Heterozygous carriers for the variants C1236T, rs2235046 and G2677T/A showed a lower risk of developing UC compared with homozygotes. Subgroup analysis revealed that C1236T and rs3789243 are associated with IBD when stratified for age of onset. The *MDR1* variant rs3789243 was found to be associated with pancolitis in UC patients[94]. In the genetically heterogeneous North Indian UC cohort was found that this SNP is significantly overrepresented in UC patients.

When German IBD patients were genotyped for the two *MDR1* SNPs in positions 2677G>T/A and 3435C>T it was found that the combined genotypes derived from these positions are possibly associated with young age onset of UC and severe course of disease[95]. The 2677T allele was significantly increased in British UC cases compared with controls. The TT genotype was significantly associated with severe UC. No significant association was seen with C3435T and UC or any clinical subgroup. A meta-analysis of 9 association studies of C3435T showed a significant association of the 3435T allele with UC, but not with CD. These results indicated that *MDR1* sequence variants are associated with a small increase in the risk of developing UC and may influence disease behavior[96]. The *MDR1* gene polymorphism G2677T/A showed significant association with CD, and the C3435T with Spanish UC patients[97]. The *MDR1* 3435 TT genotype and T-allelic frequencies were significantly higher in patients with UC compared with controls. The association was strongest with extensive UC, and this was also confirmed with multivariate analysis. However G2677T was not associated with UC or CD. Two-locus haplotypes showed both positive (3435T/G2677 haplotype) and negative (C3435/2677T haplotype) associations with UC. Homozygotes for the haplotype 3435T/G2677 were significantly increased in UC. Allelic variations of the *MDR1* gene determined the disease extent as well as susceptibility to UC in the Scottish population[90].

Nucleotide-binding oligomerization domain1/ caspase activation recruitment domain 4

Nucleotide-binding oligomerization domain1/ caspase

activation recruitment domain 4 *(NOD1/CARD4)* is a member of the Nod-like receptor family, which is phylogenetically conserved[5, 98]. It is constitutively expressed in epithelial cells throughout the gastrointestinal tract[99]. *NOD1/CARD4* contains leucine-rich repeat (LRR) domain and NOD domains and has only one CARD domain[100].

Polymorphism in LRR domain of the *NOD1/CARD4* gene showed association with disease severity of UC in North Indian patients. This might be due to disruption of the LRR region critical for NOD1-mediated bacterial sensing. Haplotype-based approach showed that GTTG haplotype carriers were over represented in UC patients which could increase the risk of the disease[101].

Initially, it was suggested that there is association of the deletion variant of *NOD1/CARD4* +32656 (complex intronic insertion-deletion polymorphism) with susceptibility to IBD using a combination of transmission disequilibrium testing (TDT) and case-control analysis[102]. However this variant was not associated with a strong effect on susceptibility to IBD in children and adults in a Northern Europe study cohort[103]. Similar results have been found in the East Anglia IBD cohort, where no association was found between *NOD1* +32656 and IBD and also no heterogeneity between UC and CD[104].

Toll-like receptors

Essential components of innate immunity are the Toll-like receptors (TLRs). These are transmembrane receptors which recognize the microbial compounds from different bacteria, fungi and viruses[105-107]. TLRs are expressed by intestinal epithelial cells and immune cells in IBD patients[108,109]. TLR signaling in the intestinal sites of the colon can inhibit the inflammatory responses and maintains the colonic homeostasis[110-112]. TLRs can be found on the cell membrane (TLR1, 2, 4, 5 and 9) or on intracellular organelles (TLR3, 7 and 8)[113]. From the 10 human TLRs we review only three members regarding to their association to UC.

TLR2 is localized on the cell's surface. With its cofactors (TLR1 and TLR6) it binds lipoproteins, which are important surface antigen of the Gram-negative outer membrane[114], TLR4 consists of a leucine-rich repeat region (LRR) and an intracellular domain homologous to IL-1 receptor[115]. It recognizes conserved pathogenic motifs of Gram-negative bacteria, mainly lipopolysaccharides (LPS). Signaling through TLR4 results in the activation of the transcriptional activator, known as nuclear factor κB (NF-κB)[116]. Similarly to TLR2, TLR9 is localized on the cell's surface. It recognizes unmethylated CpG DNA in bacteria and viruses[117,118].

The allele and carrier frequencies of the Thr399Ile mutation in the *TLR4* gene were significantly associated with UC in a Caucasian population[119]. Association of *TLR4* Asp299Gly polymorphism with UC was reported first in Caucasian UC patients[120]. In a study, mentioned before[119], increased frequency of this polymorphism was observed, but it did not reach statistical significance. Similarly to Török *et al* study, the Asp299Gly and Thr399Ile mutations in *TLR4* gene were associated with UC in Greek and in North Indian patients[121,122], but not in Dutch or Italian patients[123,124]. Interestingly, the *TLR4* Asp299Gly did not show association with UC in different Asian UC populations[125-128].

The *TLR2* Arg677Trp and Arg753Glu, *TLR4* Asp299Gly and Thr399Ile, and *TLR9* gene C1237T polymorphisms were genotyped in Chinese Han IBD patients; however none of these polymorphisms was associated with IBD. In Caucasians, both TLR4 299Gly and 399Ile conferred as a significant risk factor for developing UC and CD[127].

Three SNPs of *TLR9* (C-1486T, G1174A, A2848G) were genotyped. These variations were associated with an increased risk of UC in the Japanese population. *TLR9* -1486CC, 1174GG and 2848AA showed increased risk for UC, but *TLR9* -1486TT, 1174AA and 2848GG decreased the risk of UC, although there were no correlations between SNPs and disease phenotype or *TLR9* mRNA expression[129]. Possible associations between genetic variations in *TLR9* and IBD in the German population were investigated, but no associations were detected between *TLR9* gene variations and UC susceptibility[130].

Cell adhesion molecules

Cell adhesion molecules (CAM) mediate the extravasation of leukocytes and their accumulation in inflamed intestinal mucosa. This process is controlled by a family of CAM including the intercellular cell adhesion molecule (ICAM-1), the platelet endothelial cell adhesion molecule (PECAM-1), the selectins (E, L, and P selectin) and the integrins[131,132].

ICAM-1 (CD54) is a cell surface glycoprotein belonging to the immunoglobulin superfamily. It plays key role in transendothelial migration of leukocytes, and lymphocyte activation. The membrane glycoprotein PECAM-1 (CD31) is expressed on vascular endothelial cells, platelets, some lymphocyte subsets, and monocytes[133-135]. It has important role in transendothelial migration of circulating leukocytes during inflammatory process[136], apoptosis[137] and integrin regulation[138]. The E-selectin (CD62E) is a glycoprotein, which is expressed on endothelial cells in response to pro-inflammatory cytokines (IL-1, TNF). It supports rolling of leukocytes at sites of inflammation and tissue injury. E-selectin expression is upregulated both in CD and in UC patients playing an important role in mediating of the inflammatory process in IBD[139]. L-selectin (CD62L) is expressed on normal naive T and B cells, leukocytes and on natural killer (NK) cells. It is involved in the adhesion of T cells to endothelial cells, which are regarded as crucial in the selective migration of lymphocytes to inflamed tissue sites during an inflammatory response[140].

Several mutations in *ICAM1* (G241R and K469E), *PECAM-1* (V125L), *PECAM-1* (G98T and S128R),

E-selectin (L554F) and *L-selectin* (F206L) were analyzed in Tunisian IBD patients and controls. A significant increase in allele frequencies of 206L of *L-selectin* and the associated genotype F/L was observed both in UC and in CD patients. In the subgroup analysis the L206 allele and F/L206 genotype frequencies were significantly increased in UC patients with left-sided type. No significant differences in allele or genotype frequencies were observed for *ICAM-1*, *E-selectin*, and *PECAM-1* polymorphisms between UC patients, CD patients, and controls[141].

INTERLEUKINS IN UC

Interleukin 1

Interleukin 1 (IL-1) is pro-inflammatory cytokine, which affects cell proliferation, differentiation, and the function of many innate and specific immunocompetent cells, and acts as an endogenous pyrogen. It broadcast many inflammatory diseases by initiating and potentiating immune and inflammatory responses[142].

IL-1 is composed of two main proteins the IL-1A and the IL-1B[143]. IL-1B has major role in initiating and amplifying the inflammatory response[144]. The IL-1 receptor antagonist (IL-1RN) is an anti-inflammatory cytokine, which lacks the IL-1 receptor accessory protein (IL-1RAP) interacting domain[142].

In Mexican Mestizo UC patients five SNPs were analyzed; the rs419598, the rs315951 and the rs315952 in the *IL-1RN* gene, the rs16955 in the *IL-1B* gene and the 3811058 in the *IL-1F10* gene. Significant increased frequencies of *IL-1RN6*/1TC (rs315952), *IL-1RN6*/2CC (rs315951) and decreased frequency of *IL-1B*-511 TC (rs16944) genotypes were found in UC patients. The patients group showed increased frequencies of *IL-1RN* CTC and TCG haplotypes, whereas TTG and CTG haplotypes frequencies were decreased[145].

IL-2

IL-2 functions as a T cell growth factor, furthermore it supports the proliferation and differentiation of NK cells to increase their cytolytic functions. This IL plays important role in the development of Th1, Th2, Treg, and Th17 differentiation[146].

In the *IL-2/IL-21* region several polymorphisms (rs6822844, rs13151961, rs13119723 and rs6840978) were studies. In a Dutch population the minor alleles of the examined SNPs were associated with IBD. The strongest association of these SNPs was found in the UC patients. In an Italian UC cohort the same strong association of the minor alleles was observed with UC. Similarly to this results, in the North American study was demonstrated, that these alleles have the strongest effect among the IBD patients in the UC subgroup [147].

IL-6

IL-6 is a multifunctional, pleiotropic cytokine that is responsible for regulation of immune responses, acute-phase responses, hematopoiesis, and inflammation[148].

An Irish population study the *IL-6* -174 genotype frequency showed significant difference between CD and UC group[149]. In the Caucasian population the same polymorphism was examined in CD and UC patients and found significant difference UC and CD susceptibility[150].

IL-8

IL-8 is member of the CXC chemokine family[151]. Its has two receptors the CXCR1 (IL-8RA) and the CXCR2 (IL-8RB)[152]. It exerts effect mainly on the chemotaxis and migration of neutrophils, monocytes, lymphocytes, and fibroblasts[153].

IL-8 T-251A was analyzed in a Polish population. The allele frequency showed significant difference comparing the UC group to the controls[154], however this association was not observable in a Chinese UC cohort[155]. Additional polymorphisms were also tested and their effect on the serum level of IL-18. Haplotype frequency of the -353A/ -251A/ +678T haplotype was considerably higher in UC group compared to controls, suggesting this haplotype is likely to be more common in severe UC patients than in mild to moderate cases[155].

IL-10

The anti-inflammatory cytokine IL-10 is produced by many cells like monocytes, T cells, B cells, NK cells, macrophages, and dendritic cells (DCs). It prevents the antigen presentation and also the subsequent release of pro-inflammatory cytokines, so it alleviates the activated immune system[156].

In a GWAS, the polymorphism rs3024505 demonstrated the most meaningful association in the combined verification UC samples, suggesting that defective IL-10 function plays important role in the pathogenesis of the UC[157]. The same polymorphisms was investigated in Australian population[158] and Danish cohort[159] and found that the rs3024505 was associated with the risk of UC.

Three promoter polymorphisms of the *IL-10* gene G-1082A, C-819T, and C-592A were studied in many population but the results are contrary. In an Italian cohort the G-1082A and the C-819T SNPs were investigated. The -1082 genotype frequencies were significantly different between UC patients and controls. The frequency of the -1082A allele was also significantly higher in the UC patients than in controls. Allele and genotype frequencies of T-819C were not significantly associated with the disease. Furthermore, the frequencies of haplotypes -1082A/-819C and -1082A/-819T, which have been described to have a decreased promoter activity, were significantly increased in UC patients than in controls[160]. In a North-Eastern Mexican population the G-1082A and the C-592A SNPs were examined. The -1082 AA and -592 AA genotypes showed significantly lower frequencies in UC compared to healthy controls, while individuals heterozygous at *IL-10*-1082 have sig-

nificantly increased occurrence of UC[161]. In a Tunisian group the A-627C and the G-1117A polymorphisms were examined and found that these two variants influencing the UC susceptibility and phenotype[162]. In the Asian population the association was confirmed between A-1082G polymorphism and UC[125, 163].

IL-12

IL-12 is an interleukin that is naturally produced by dendritic cells, macrophages and human B-lymphoblastoid cells in response to antigenic stimulation. It participates in the differentiation of naive T cells into Th1 cells and involved in the activities of natural killer cells and T lymphocytes. IL-12 mediates gradation of the cytotoxic activity of NK cells and CD8+ cytotoxic T lymphocytes[164]. IL-12 consist of two subunit p35 (IL-12A) and p40 (IL-12B), which is shared by IL-12 and IL-23 cytokines. The IL-12 receptor has two subunits: IL-12RB1 and IL-12RB2[165, 166].

In a German population four SNPs (rs3212227, rs17860508, rs10045431 and rs6887695) of the *IL-12B* were investigated. Two SNPs, the rs10045431 and rs6887695 showed association with increased UC susceptibility[167]. From these SNPs the rs6887695 was investigated in a Japanese population where significant association was manifested between UC patients and controls[168].

IL-17

The interleukin 17 (IL-17 or IL-17A) is a pro-inflammatory cytokine secreted by activated T cells its main tasks is inducing and mediating pro-inflammatory responses. It induces the production of many other cytokines, chemokines and prostaglandins from fibroblasts, endothelial cells, epithelial cells, keratinocytes, and macrophages[169-171].

In a Japanese population the rs2275913 SNP in the *IL-17A* gene and the rs763780 SNP in the *IL-17F* gene were investigated and found significant differences between UC group and healthy controls on level of -197A/A and 7488T/T genotype frequencies[172].

Even more recently, a GWAS in a very large European UC cohort identified an association between another IL-17 pathway gene (*IL-17REL*) and UC[173].

IL-18

IL-18 is produced by macrophages and other cells. It functions by binding to the interleukin-18 receptor, and together with IL-12 it induces cell-mediated immunity following infection. IL-18 induces gene expression and synthesis of TNF, IL-1, Fas ligand, and several chemokines[174].

In the *IL-18* gene several polymorphisms were examined. The G-137C, the C-607A and the G-656T are located in the promoter region, while the A105C, the T113G and the C127T are coding variants. In a Japanese study the G allele at 113 and the T allele at 127 were significantly higher in patients with UC compared to the control[175]. In another Japanese study allele and genotype frequency of G-137C were significantly higher in the proctitis-type UC patients than in controls[176]. The frequency of haplotype 2 (-607A, -137C), which have lower promoter activity and IFNγ-mRNA level was significantly increased in the proctitis-type patients than in the control group[176]. The C-607A and the G-137C SNPs were associated with the development of UC in Tunisian patients. The -137GG genotype frequency was significantly higher in UC than in controls and statistically significant association was found between -607AA genotype in UC patients and the distal localization of the lesions[177].

IL-23

IL-23 main functions are very important in innate and adaptive immunity to regulate Th17 function and expansion[178]. This cytokine induces CD8+ memory T cells to proliferate and produce IL-17. IL-23 binds to its receptor IL-23R, which polymorphisms' play the main role in the autoimmune diseases[179-181] especially in IBD[182].

Several independent functional SNPs of the *IL-23R* gene and its neighboring region were determined; several were found susceptible to (rs10889677, rs11209032, rs11465804, rs11805303, rs1495965, rs2201841, rs1004819) CD and UC in non-Jewish subjects[183].

In a Chinese cohort the rs7530511 and rs11805303 SNPs were studied and positive association was found between these variants and UC susceptibility[184]. In the Jiangsu Han population the rs11805303 was found as a susceptibility factor to UC[185].

In a Swedish population the rs10889677, the rs11209032, the rs11465804, the rs2201841 and the rs1004819 polymorphisms were investigated, and found that the rs11465804G, rs2201841C and rs1004819T allele frequencies showed significant differences between UC patient group and control. These genetic variants are individual risk factor for developing the disease[186].

In Hungarian UC patients for the *IL-23R* rs1004819A allele we found significantly higher allele frequency compared to control subjects and the SNP rs2201841 showed significant association with UC risk for homozygotes[187].

IL-26

Expression of IL-26 seems to be restricted to memory T cells, NK cells, and Th17 cells. Thereby it could have pro-inflammatory effects in IBD[188].

Only a few markers were investigated, from these the rs2870946-G and the rs1558744-A showed association with UC[189]. Further meta-analysis study confirmed the association of rs1558744-A with UC[190].

CONCLUSION

This review shows that substantial progress has been made in the past 10 years in to find inflammatory related genetic factors and cytokines in UC. We reviewed different genes and gene polymorphisms which play role in the inflammatory process of UC. These genes could be

potential targets of novel treatment strategies.

From the reviewed genes contributing to inflammation *TNFα*, *MDR1* and *TLRs* were the most investigated genes. *TNFα* has been found as a susceptibility locus for both UC and CD[34-36]. The *TNF2* allele and *TNF 1/2* could be good candidate markers for the susceptibility to UC. Based on the different studies with different populations (East Asians, Han Chinese, Spanish Caucasoid, Italian, and Czech), the *TNFα* -308 is the most studied SNP and this may be an ethnic population-specific risk factor for UC especially for Asian populations but not for Europeans. It should be noted this polymorphism is also a susceptibility factor in other autoimmune disorders (asthma, psoriasis, and rheumatoid arthritis) too.

The *MDR1* C3435T is one of the most tested SNP in UC, but with conflicting results. Some studies showed significantly increased 3435T allele and 3435TT genotype frequencies of C3435T[81,83,97], or only a trend towards an increased frequency of T allele[82], or the T allele was predictive of susceptibility to later onset UC, but not for the early onset of UC[89]. But some studies failed to demonstrate association with UC[91-93]. Other SNPs of *MDR1* (C1236T and rs3789243) were associated with IBD when stratified for age of onset. The rs3789243 was found to be significantly overrepresented in genetically heterogeneous North Indian UC patients.

We reviewed 3 members from the 10 human TLRs regarding to their association to UC. Allele and carrier frequencies of the *TLR4* Asp299Gly and Thr399Ile were significantly associated with UC in Caucasian[119,120], Greek and in North Indian patients[121,122], but not with Dutch, Italian[123,124] or Asian patients[125-128]. Interestingly the *TLR9* polymorphisms (C-1486T, G1174A, A2848G) were associated with an increased risk of UC in the Japanese population. *TLR9* -1486CC, 1174GG and 2848AA polymorphisms show increased risk for UC, but *TLR9* -1486TT, 1174AA and 2848GG decrease the risk of UC[129].

From the reviewed cytokines *IL-10, IL-18* and *IL-23* were the most investigated genes. The IL-10 is a major anti-inflammatory cytokine, which attenuates the activated immune system with inhibiting both the antigen presentation and subsequent release of pro-inflammatory cytokines. IL-10 is a shared risk gene for CD and UC too. The promoter polymorphisms of this gene (G-1082A, C-819T, and C-592A) which are in tight linkage disequilibrium were extensively studied in many populations but with contradictory results. In the Caucasian population the carriers of G-1082A SNP were more susceptible to UC, whereas in another study carriers were associated with lower UC incidence[160,161]. In the Asian population the results strengthened the positive relationship between this SNP and UC susceptibility[125,163]. In a North-Eastern Mexican population the -592AA genotypes showed significantly decreased frequency in UC compared the results to the healthy controls[161]. In a Tunisian group the A-627C and the G-1117A variants influencing the UC susceptibility and phenotype[162]. Several other studies handle with these non-coding SNP in CD too and determine susceptibility to the disease or not.

The G-137C, the C-607A and the G-656T promoter SNPs and several others in the coding regions of *IL-18* gene (A105C, the T113G and the C127T) were examined. The Japanese population is the most studies for these SNPs, significant difference was found in the allele frequency of the A105C between CD patients and controls, while this correlation could not be detected in UC patients. The 113G and 127T allele frequencies were significantly increased in patients with UC compared the results to the healthy controls[175]. In case of promoter polymorphisms, the -137CC genotype frequency was significantly increased in proctitis-type UC patients than in controls, while the other two C-607A and G-137C SNPs were associated with the development of UC in Tunisian patients[177].

The *IL23R* gene was identified as a CD susceptibility gene in North American non-Jewish subjects. Several independent functional SNPs in the gene and its neighboring region were determined[183]. After the primary publications, several studies have been published these SNPs in IBD and other autoimmune disease too (ankylosing spondylitis, psoriasis, Sjögren syndrome, systemic lupus erythematosus). From these SNPs (rs10889677, rs11209032, rs11465804, rs11805303, rs1495965, rs2201841, rs100481) several are risk factor to IBD both in European and Asian populations[185-187].

It can be established that these interleukin gene variants are strongly population dependent but in the given population they can be predictors for CD or UC. Despite the advances in the field of UC/IBD genetics, testing for these genetic variants is currently not recommended for clinical purposes[191].

Understanding of the detailed pathogenesis of IBD and identifying new disease associated SNPs led to the development of selective inhibitors for ILs, chemokines and their receptors. This strategies can optimize treatment efficacy and lead to personalized medicine based on the patient's genotype.

Biological agents are used in patients with moderate to severe disease activity who have failed conventional therapy with glucocorticoids and thiopurines. Today, the most effective and best studied anti-cytokine agents in IBD are the anti-TNFα antibodies. The mechanism of action of TNFα antagonists is based on the neutralization of both soluble TNFα and membrane TNFα and has a more global effect on inflammation than the blockade of other cytokines. Currently, three TNFα inhibitors are approved by the United States Food and Drug Administration (FDA) for inducing and maintaining clinical remission in UC: the chimeric (25% murine and 75% human sequence) monoclonal full-length IgG1 mAb infliximab[192], the fully human mAbs adalimumab[193,194] and golimumab[195]. The pegylated humanised antibody certolizumab pegol is approved only for CD (beside rheumatoid arthritis, RA and psoriatic arthritis). Etanercept, a

dimeric fusion protein consisting of soluble p75-TNFR2 and the Fc portion of human IgG1, used in rheumatoid arthritis therapy, is not efficient for the treatment of intestinal inflammation[196].

Despite the expeditious development of newer biological therapies, only few have shown benefit in clinical trials in UC. Targeting of IL-23 or the IL-23 receptor or IL-23 axis is a potential therapeutic approach for autoimmune diseases including psoriasis, IBD, RA and multiple sclerosis[197]. Recently, testing of anti-IL-12/23 treatment in patients with CD has been performed. In Phase Ⅱ trial, patients with moderate to severe CD that was resistant to TNF antagonists had an increased rate of response to induction with the fully human mAb ustekinumab directed against the p40, as compared with placebo[198]. However, due to the common p40 subunit and IL-12RB1 chain, the major drawback of anti-IL-23 treatment can be the simultaneous inhibition of IL-12 and a possible shutdown of the immune system. Nevertheless, it would be much more useful to design drugs that target the IL-23p19 or IL-23RA itself, so inhibiting IL-23 without modifying the effects of IL-12[197].

Treatment of CD patients with the IL-17 blocker secukinumab (anti-IL-17A) was ineffective and higher rates of adverse events were noted compared with placebo[199].

One new additional treatment for UC may be tofacitinib, an inhibitor of Janus kinases 1, 2, and 3 with *in vitro* functional specificity for kinases 1 and 3 over kinase 2, which is expected to block signaling involving gamma chain-containing cytokines including ILs-2, 4, 7, 9, 15, and 21. Tofacitinib, was approved for the treatment of RA in the USA, Japan and Russia in April 2013. In a double-blind, placebo-controlled, Phase Ⅱ trial, patients with moderately to severely active UC treated with tofacitinib were more likely to have clinical response and remission than those receiving placebo[200].

Targeting leukocyte recruitment and cell adhesion molecules could be also an option for IBD therapy. Natalisumab, a recombinant humanised monoclonal IgG4 antibody, targets both the α4β1 heterodimer located in the central nervous system and the α4β7 integrin in the gut. The FDA approved natalizumab for both induction of remission and maintenance of remission for moderate to severe CD, though it has not been approved for this use in the European Union due to concerns over its risk/benefit ratio (risk of progressive multifocal leukoencephalopathy)[201]. Vedolizumab is a humanized mAb that specifically recognizes the α4β7 heterodimer, selectively blocks gut lymphocyte trafficking without interfering with trafficking to the central nervous system. In the Phase Ⅲ study, vedolizumab was more effective than placebo as induction and maintenance therapy for UC suggesting that blockade of T cell homing in the gut may favor mucosal healing in UC[202, 203].

The exact positioning of these promising new therapies in the management of UC remains uncertain currently. Additional long-term safety data and clinical experience will be needed to determine an overall benefit/harm ratio of newly developed biological agents.

The identified separate loci in IBD research individually have only modest effects on IBD susceptibility. They account together for only 20%-25% of the heritability, suggesting that gene-gene interactions as well as gene-environmental interactions could play a key role in IBD pathogenesis and fill the so called "genetic vacuum" of polygenic diseases[204]. More complete understanding of the immunopathogenic role of the various genes and ILs in intestinal inflammation will help in the development of more effective novel therapeutic strategies in UC. Next generation techniques in combination with the data analysis by systems-biology approach hopefully will contribute to the personalized therapy of the patients in the near future.

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REVIEW

Current status of predictive biomarkers for neoadjuvant therapy in esophageal cancer

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Abstract

Neoadjuvant therapy has been proven to be extremely valuable and is widely used for advanced esophageal cancer. However, a significant proportion of treated patients (60%-70%) does not respond well to neoadjuvant treatments and develop severe adverse effects. Therefore, predictive markers for individualization of multimodality treatments are urgently needed in esophageal cancer. Recently, molecular biomarkers that predict the response to neoadjuvant therapy have been explored in multimodal approaches in esophageal cancer and successful examples of biomarker identification have been reported. In this review, promising candidates for predictive molecular biomarkers developed by using multiple molecular approaches are reviewed. Moreover, treatment strategies based on the status of predicted biomarkers are discussed, while considering the international differences in the clinical background. However, in the absence of adequate treatment options related to the results of the biomarker test, the usefulness of these diagnostic tools is limited and new effective therapies for biomarker-identified nonresponders to cancer treatment should be concurrent with the progress of predictive technologies. Further improvement in the prognosis of esophageal cancer patients can be achieved through the introduction of novel therapeutic approaches in clinical practice.

Key words: Esophageal cancer; Neoadjuvant therapy; Response prediction; Molecular biomarker; Chemoradiation

Core tip: To achieve individualization of neoadjuvant therapy for locally advanced esophageal cancers, predictive biomarkers are urgently needed. Biomarker development using multimodal approaches, including gene expression profiling, single nucleotide polymorphisms, microRNAs, proteomics, immunohistochemistry, serum biomarkers and conventional blood tests, seem promising. Independent validation studies will establish novel prognostic modalities based on molecular biomarkers. Progress of predictive modalities and further studies on the molecular background of patients with a poor prognosis will facilitate the development of new effective therapies for patients resistant to the present neoadjuvant therapy. Prognostic stratification of patients will promote efforts toward novel therapeutic strategies.

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INTRODUCTION

Esophageal cancer is the fifth most common cause of cancer-related death for men and the eighth for women worldwide[1]. Despite the use of modern surgical tech-

niques in combination with radio- and chemotherapy, early recurrence is common and the overall 5-year survival rate remains below 40%[2]. Consequently, there is a great interest in multimodal approaches to the treatment of esophageal cancer and neoadjuvant chemotherapy, alone or in combination with chemoradiotherapy (CRT), is becoming the standard approach of care in locally advanced esophageal cancers. Randomized trials of different neoadjuvant therapy protocols have been conducted in patients with locally advanced cancers. Meta-analyses of those randomized trials have revealed only modest survival advantages, except in the case of patients who achieved a complete histopathological response and seemed to highly benefit from a neoadjuvant regimen[3-9]. However, a significant proportion (60%-70%) of treated patients did not respond well to these treatments and experienced severe adverse effects[8,10]. In addition, nonresponsive patients may lose the option of surgical resection after ineffective chemotherapy[11] and the prognosis of nonresponders has been found to be inferior to that for patients treated by surgery alone[12]. While there is an obvious correlation between the response and prognosis, the response to chemotherapy or radiotherapy is variable, even when patients are at the same clinical stage. Thus, an accurate risk stratification of cancer patients for therapy is of paramount importance for avoiding potential morbidity due to ineffective treatment and prevention of further disease progression. With this background, identification of predictive markers would allow accurate risk stratification and individualization of multimodality treatment for patients with locally advanced esophageal cancer[13].

In recent years, molecular biomarkers that can predict the response to neoadjuvant therapy in esophageal cancer have been investigated by using multidimensional approaches. Global expression transcriptomics and proteomics studies allow for simultaneous screening of several thousand molecules and knowledge-based methodologies such as immunohistochemistry are focused on a specific molecule or pathway. These approaches are based on their own unique principles and the performance of predictive molecular biomarkers developed by using each approach seems to be equally promising. Here, we have reviewed the current status of molecular biomarkers predictive for response to neoadjuvant therapy in esophageal cancer. We have focused on predictive markers that can be used to analyze pretreatment samples such as diagnostic biopsies or serum specimens obtained before neoadjuvant treatment. These biomarkers will help avoid unnecessarily invasive treatments. We have summarized promising candidates for predictive molecular biomarkers in esophageal cancer according to the type of development modality.

MOLECULAR BIOMARKERS FOR RESPONSE PREDICTION

Gene expression profiling

High throughput technology such as gene expression microarray has been considered as one of the most powerful tools for understanding the biological characteristics of malignancies. Microarray-based gene expression profiling generates quantitative expression data for thousands of genes, which can be further analyzed by various bioinformatics approaches to identify the most informative genes relevant to cancer prognosis. In particular, the gene expression signatures determined by microarrays have been used to predict the response to neoadjuvant treatment among cancer patients[14].

Maher *et al*[15] investigated gene expression profiles in a cohort comprising 13 patients who were the most responsive or resistant to a standard combination of chemotherapy and radiation therapy. The authors identified five genes (*EPB41L3*, *RNPC1*, *RTKN*, *STAT5B* and *NMES1*) as predictive biomarkers by using DNA microarrays and validated the results by qRT-PCR, confirming that the expression level of five genes could be used to predict the response to neoadjuvant CRT in esophageal cancer with 95% accuracy. Luthra *et al*[16] profiled pretreatment endoscopic cancer biopsies from 19 patients using an AffymetrixU133A Chip (Santa Clara, CA) and noted correlation of the molecular profiles with pathological response to neoadjuvant treatments. The authors reported that the expression levels of three genes (*PERP*, *S100A2 and SPRR3*) helped discriminate between patients with complete histopathological response and those resistant to treatment, with high sensitivity (86%) and specificity (85%). Schauer *et al*[17] performed microarray analysis in 47 patients who had a locally advanced esophageal adenocarcinoma (AC) and had undergone neoadjuvant chemotherapy with cisplatin, leucovorin and 5-fluorouracil, followed by resection. The authors found that the gene encoding the ephrin B3 receptor showed the most prominent differential expression between responders and nonresponders and validated these results by immunohistochemistry. Motoori *et al*[18] performed comprehensive gene expression profiling of pretreatment biopsy samples from 25 patients with esophageal squamous cell carcinoma (SCC) to identify expression patterns predictive for cisplatin-based neoadjuvant chemotherapy. Their system consisted of 199 most informative genes and had the prediction accuracy of 82%. Duong *et al*[19] performed microarray analysis for 46 esophageal cancer patients, that is, 21 SCC and 25 AC patients for whom neoadjuvant CRT had been recommended. Their study was based on two-color competitive hybridization to a cDNA array printed at the Peter MacCallum Cancer Centre Microarray Core Facility[19] and identified a 32-gene classifier that could be used to predict a response to neoadjuvant CRT in SCCs, whereas a negative predictive profile was observed for AC patients.

These examples suggest that gene expression profiling is a powerful tool to identify gene sets for selection of optimal and personalized therapy for patients with esophageal cancer. In breast cancer, mRNA expression signatures strongly predictive of metastasis have been identified and a novel prognostic test for assessing the

risk of metastasis and benefits of chemotherapy has been introduced in clinical settings. This test, named MammaPrint, effectively identifies breast cancer patients with a high risk of recurrence after local treatment alone[20]. The Oncotype DX assay (Genomic Health, Redwood, CA) is another test aimed at better discerning breast cancer patients who would benefit from chemotherapy and those who can safely avoid it. By using the Oncotype DX, we measured the status of 21 genes and could predict the benefits of chemotherapy and the rate of cancer recurrence in 10 years[21]. Similar diagnostic predictive tests are desired for esophageal cancer; however, in this case, different prognostic biomarkers have been identified by using similar technical platforms. The results of these studies need further validation in order to forward their clinical application.

Single nucleotide polymorphisms

In the process of generating a draft sequence of the human genome, it has become clear that the extent of genetic variation is much larger than previously estimated[22,23]. The most common sequence variation in the human genome is the stable substitution of a single base called single-nucleotide polymorphism (SNP). By definition, SNP has a minor allele frequency of greater than 1% in at least one population[24]. Most SNPs are silent and do not alter gene expression or function. The cancer genomics research on SNP variation provides an opportunity for the detection of molecular biomarkers predictive of the response to cancer therapy[25].

Wu *et al*[26] investigated the association between SNPs in multigenic cascades involved in radiation and chemotherapy-dependent responses and clinical outcomes for esophageal cancer patients. The authors applied the pathway-based approach to examine the impact of a comprehensive SNP panel on clinical outcomes in 210 esophageal cancer patients and found that among the genes involved in DNA base excision repair, the variant alleles R399Q in the *XRCC1* gene were significantly associated with the absence of complete pathological response and poor survival. Warnecke-Eberz *et al*[27] investigated a panel of selected gene SNPs to predict responses to neoadjuvant radiochemotherapy in 52 esophageal cancer patients. The authors showed that SNP of C118T in the *ERCC1* gene and the rarely occurring AA genotype of the *XRCC1* gene were predictive of therapy response. Both *ERCC1* and *XRCC1* genes are components of the nucleotide excision repair pathway that protects the integrity of the genome by removing a wide variety of DNA lesions including inter- and intra-strand crosslinks caused by platinum agents or radiation[28]. These SNPs in *ERCC1* appeared to have functional significance because a low intra-tumoral expression of the ERCC1 protein was found to be strongly associated with a major pathological response[29,30]. Moreover, Brabender *et al*[31] reported that *ERCC1* RNA expression in peripheral blood could be a predictor of the response to neoadjuvant therapy. Functional contribution of SNPs in other genes involved in nucleotide excision repair should be investigated for further understanding of the pathogenesis of esophageal cancer.

Clinical applications of SNP testing in cancer are quite realistic. In other types of cancer, the cancer genomics research on SNP variation has provided clinical applications. For example, genetic polymorphisms of the *UGT1A1* gene would affect inter-individual variations in the toxic response to irinotecan by altering the bioavailability of the irinotecan active metabolite SN-38[32,33]. Genetic testing for the presence of the UGT1A1*28 allele has been approved by the FDA and has become available in hospitals. Similar tests for genetic polymorphisms in esophageal cancer would be extremely useful and validation studies for the predictive potential of SNPs would promote their introduction in clinics.

MicroRNAs

MicroRNAs (miRNAs) are short (19-24 nucleotides) noncoding RNA sequences involved in the regulation of gene expression *via* the inhibition of mRNA translation[34,35]. Many lines of evidence suggest that miRNAs exist stably in tissues and body fluids and play a key role in various biological processes, including carcinogenesis. Aberrant miRNA expression has been shown to correlate with the inhibition of tumor suppressor genes or inappropriate activation of oncogenes. Recent studies have shown that the abnormal miRNA expression patterns frequently detected in esophageal cancers have strong prognostic values[36-39]. The predictive utility of miRNAs has also been demonstrated by global expression studies.

Odenthal *et al*[40] assessed miRNA profiles of responders and nonresponders to neoadjuvant therapy for esophageal cancer in order to identify possible predictive markers. The authors found that the pre-therapeutic intra-tumor expression of miR-192 and miR-194 was significantly associated with the histopathological response of esophageal SCCs to multimodal therapy. Using pretreatment biopsy specimens, Ko *et al*[41] showed that the miRNA expression profile was significantly different between groups with and without complete pathological response. Among the 71 differentially regulated miRNAs, five showed the difference of more than two-fold; these included miR-296[42], which has recently been shown to be of prognostic significance in esophageal cancer. The inhibition of miR-296 also resulted in the increased chemosensitivity of esophageal cancer cells to standard chemotherapeutic agents such as 5-fluorouracil and cisplatin[42]. Tanaka *et al*[43] investigated the serum levels of miR-21, miR-145, miR-200c and let-7c by qRT-PCR in 64 esophageal cancer patients treated with neoadjuvant chemotherapy. The authors revealed a significant correlation of miR-200c high expression with poor response to chemotherapy. The possible prognostic utility of miR-200c was also reported by Hamano *et al*[44], who in a study of 98 patients found that miR-200c was involved in resistance to chemotherapy. Lynam-Lennon *et al*[45] demonstrated that resistance to radiation was sig-

nificantly associated with the downregulation of miR-31 and that the ectopic re-expression of miR-31 considerably restored radiosensitivity of the resistant cells. The authors also showed that miR-31 expression was markedly reduced in patients with poor pathological response to neoadjuvant CRT, whereas the expression of the miR-31-regulated DNA repair genes significantly increased[45].

Clinical application of miRNAs as predictive biomarkers is quite feasible because miRNAs are relatively stable and their expression levels can be quantitatively assessed by qRT-PCR. Currently, several clinical trials have already been approved by the FDA to evaluate the value of serum miRNAs in therapeutic response prediction (http://clinicaltrials.gov). Clinical trials evaluating serum miRNAs include the search for predictors of therapeutic response in ovarian carcinoma and miRNA profiling of breast cancer in patients undergoing neoadjuvant or adjuvant treatment[46]. Further functional studies would hopefully validate the functional relevance of miRNAs in esophageal cancer and result in diagnostic and novel therapeutic approaches.

Proteomics

The proteome is a functional translation of the genome. The genomic aberrations in cancer cells are translated to the proteome determining cancer phenotypes and regulating tumor behavior. Because proteins are the main executioner biomolecules, which influence the molecular pathways in normal and tumor cells, proteomic markers are closer and more relevant to cancer initiation and progression than other biomarkers. Proteomic studies can therefore generate unique data related to cancer phenotypes. Many lines of evidence have demonstrated the discordance between mRNA and protein expression[47-49]. In addition, DNA sequence and mRNA expression cannot accurately predict post-translational modifications such as phosphorylation and glycosylation, which play a key role in regulating the malignant behavior of cancer cells. Taken together, proteomic studies can provide valuable information for biomarker identification in various cancers[50-52].

Aichler *et al*[53] analyzed proteomic changes associated with response to chemotherapy by MALDI imaging mass spectrometry using pre-therapeutic biopsy samples of 23 esophageal ACs. Proteins related to clinical response were identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The authors discovered that clinical response to cisplatin was associated with the defects in the mitochondrial respiratory chain of cancer cells caused by the loss of specific cytochrome c oxidase subunits. Maher *et al*[54] examined the proteomic profiles of serum samples by using surface-enhanced laser desorption/ionization time-of-flight (SELDL-TOF) mass spectrometry and validated the results with an enzyme-linked immunosorbent assay. By comparing pre-treatment serum samples from 16 poor responders and 15 good responders, the authors found that higher serum levels of complement factors C4a and C3a were significantly associated with favorable response to treatments. The leave-one-out cross-validation analysis revealed that these serum proteins could predict the response to neoadjuvant CRT with a sensitivity and specificity of 78.6% and 83.3%, respectively.

Although there are various reports about biomarker candidates identified by proteomics studies, only a few of them have been proven to be clinically useful[55] because of the lack of independent validation studies. However, the prognostic utility of protein biomarkers has been successfully validated for gastrointestinal stromal tumors in extensive multi-institutional studies[56]. Further validation studies will promote the clinical application of promising protein biomarkers for esophageal cancer.

Immunohistochemistry

By focusing on functionally important molecules or pathways, discovery of biomarker candidates can be performed effectively. Global expression studies based on statistical data may not be able to identify functionally important genes and proteins because expression levels do not always reflect functional activity. In this sense, a knowledge-dependent approach such as immunohistochemistry has unique advantages over the other methods for expression assessment because it allows for the analysis of a large number of formalin-fixed and paraffin-embedded tissue sample archives and provides detailed spacious information not available by other methods. Immunohistochemistry has been successfully used for hypothesis-driven biomarker discovery[57].

Solid tumors are driven and managed by a small population of cancer stem cells (CSCs), tumor-initiating cells or cancer stem-like cells[58-61]. Among these cells, CSCs are found to be more resistant to treatment[62,63]; therefore, CSC markers have been considered promising candidates for predictive biomarkers. Previous reports have demonstrated the importance of CSC markers including growth factor receptors, tumor suppressor genes and DNA-repair pathway factors in malignant features of esophageal cancer cells. Smit *et al*[64] investigated the expression of CSC markers, *in vitro* growth of spheroids, sensitivity to radiation and *in vivo* growth of several esophageal cancer-derived cell sub-populations. The authors found that the CD44+/CD24- subpopulation of esophageal cancer cells exhibited a higher proliferation rate and sphere forming potential and was more radioresistant *in vitro* than unselected or CD44+/CD24+ cells. In a study of the archival pre-neoadjuvant CRT biopsy material from esophageal AC patients (N = 27), CD44+/CD24- cells could only be identified in 50% (9/18) of poor responders to neoadjuvant CRT, but never (0/9) in complete responders. These results warrant further investigation into the possible clinical utility of CD44+/CD24- phenotype as a predictive biomarker for the response to CRT in patients with esophageal cancer.

Human epidermal growth factor receptors 1 and 2

(EGFR and HER2/neu) are known to be involved in malignant transformation and tumor growth. Yamamoto *et al*[65] assessed the expression of EGFR, HER2/neu, HER3, Ki-67 and p53 by immunohistochemistry in 37 esophageal SCC patients treated with neoadjuvant chemotherapy and found that EGFR expression correlated with pathological response to neoadjuvant chemotherapy. Akamatsu *et al*[66] reported similar findings in 34 patients who had esophageal SCC and were receiving neoadjuvant CRT, *i.e.*, positive staining for HER2/neu was found to be associated with CRT resistance. In contrast, Arsenijevic *et al*[67] and Schena *et al*[68] found no statistically significant difference between EGFR and HER2/neu expression and the clinical response to neoadjuvant CRT. Further verification studies are necessary to clarify the role of EGFR and HER2 expression in the response of esophageal cancer patients to CRT.

The tumor suppressor gene p53, which is involved in cell cycle regulation, apoptosis and DNA repair, has been identified as an important molecular factor in the response to neoadjuvant therapy in patients with esophageal cancer[69]. However, the predictive value of p53 status for chemotherapy response in esophageal cancer patients has not been established. Kitamura *et al*[70] performed a study involving 95 patients with esophageal SCC and showed that p53 protein expression was significantly associated with increased sensitivity to neoadjuvant CRT. In contrast to these findings, Shimada *et al*[71] demonstrated that p53 protein expression was negatively associated with histopathological response to chemotherapy, whereas other similar studies did not find any predictive value for p53 in multimodality therapy for esophageal cancer[67,72]. Zhang *et al*[73] conducted a meta-analysis of 28 studies comprising 1497 cases to elucidate the correlation of p53 status with the response to chemotherapy-based treatment. The authors concluded that patients with low expression of wild-type p53 had higher rates of complete pathological response to neoadjuvant CRT. The clinical significance of p53 as a predictive biomarker for the treatment of esophageal cancer should be further evaluated.

DNA repair pathways are essential for the cell responses to DNA damage induced by CRT. Aberrant regulation of DNA repair proteins is frequently reported in cancers and the reduced expression of these proteins correlated with poor prognosis in esophageal cancers[74-76]. Alexander *et al*[77] assessed major DNA repair proteins such as XPF, FANCD2, PAR, MLH1, PARP1 and phosphorylated MAPKAP kinase 2 in 79 patients with esophageal cancer by tissue microarray. The authors showed that higher scores for MLH1 and lower scores for FANCD2 were significantly associated with pathological response to neoadjuvant CRT on multivariable analysis.

Expression of heat-shock proteins (HSPs) and glucose-regulated proteins (GRPs) can be induced in cells following exposure to different insults, allowing cells to survive stress conditions. The regulation and expression of these proteins have an important impact on the biology of esophageal cancer with respect to prognosis[78] and response to chemotherapy[79]. Slotta-Huspenina *et al*[80] assessed HSPs and GRPs by reverse phase protein arrays (RPPAs), immunohistochemistry and quantitative RT–PCR in pretherapeutic biopsies of 90 patients with esophageal AC. The authors showed that low expression of HSP90, HSP27 and p-HSP27$^{(Ser15, Ser78, Ser82)}$ and high expression of GRP78, GRP94, HSP70 and HSP60 were significantly associated with pathological response to neoadjuvant chemotherapy.

Even with the advances in modern technologies, the emergence of new biomarkers for esophageal cancer has been relatively slow because biomarker discovery has been generally hypothesis-driven and depended on investigation of individual genes or proteins. Data-driven approaches such as global expression studies provide a considerable number of biomarker candidates and once their functional and clinical significance is established, they are worth validating by immunohistochemistry. Immunohistochemistry is an established clinical examination method and further validation studies on biomarker candidates confirmed by immunohistochemistry should be relatively easily performed. A possible utility of these candidate proteins as predictive biomarkers for neoadjuvant CRT should be further validated.

Serum biomarkers with response to treatments

The hypothesis-driven approach is used to examine serum proteins, which have been previously established as biomarkers but have not been considered as predictive biomarker candidates. Serum samples can be obtained by a minimally invasive procedure at a relatively low cost and thus can be repeatedly examined. There are several reports that conventional serum biomarkers could be predictive in esophageal cancer.

Makuuchi *et al*[81] examined the expression levels of 84 cytokines in serum samples obtained from 37 esophageal SCC patients treated with neoadjuvant CRT. They found that the level of serum soluble IL-6 receptor was significantly higher in 30 patients who failed to achieve a complete histological response, thereby revealing a correlation between serum IL-6 receptor levels and the histological response to neoadjuvant CRT. These observations suggest that persistent systemic inflammation can be a possible mechanism of resistance to CRT therapy in esophageal cancers.

Brabender *et al*[82] assessed thymidylate synthetase and dihydropyrimidine dehydrogenase RNA expression in the peripheral blood of 29 patients who had esophageal cancer and had been treated with neoadjuvant CRT. The authors showed that high thymidylate synthetase expression was associated with a minor response to neoadjuvant treatment, while there was no significant association between dihydropyrimidine dehydrogenase and treatment response. They also reported that the specificity of response prediction reached 100% when the levels of thymidylate synthetase and dihydropyrimidine dehydro-

genase were assessed simultaneously.

Only a few serum biomarkers have been examined for predictive utility in cancers and it is challenging to investigate the rest of them. Such an examination does not require significant sample volumes and it is quite feasible to examine multiple serum biomarkers in identical cohorts. Serum biomarkers can be routinely examined in the clinical setting and their application to the prediction of treatment responses seems to be quite promising.

Common blood tests

Data obtained by common blood tests can be an indicator of response to neoadjuvant therapy. It is noteworthy that, although serum examination may lack specificity and sensitivity, its combination with common blood tests can provide predictive stratification of esophageal cancer patients for chemotherapy.

Sato *et al*[83] investigated the correlation between the pre-therapeutic neutrophil to lymphocyte ratio (NLR) and pathological response to neoadjuvant chemotherapy in patients with advanced esophageal cancer. The authors showed that the pretreatment NLR ($< 2.2/ \geqslant 2.2$) was significantly correlated with pathological response: the pathological response rates were 56% and 21% in patients with the NLR < 2.2 and NLR > 2.2, respectively. Similar results were reported by Noble *et al*[84], who examined the correlation of blood-borne inflammatory and nutritional markers with response to neoadjuvant chemotherapy in radically treated esophagogastric cancer patients. The authors demonstrated that only serum albumin ($P = 0.037$) had a predictive value for the pathological response to chemotherapy and that a higher NLR was associated with poor overall survival. In contrast, Hsu *et al*[85] reported that none of the clinical parameters, including blood profiles, images and baseline tumor characteristics, predicted the response to CRT.

Cancer always unfolds on a background of chronic inflammation and it is an interesting idea that inflammatory markers can also serve as prognostic biomarkers for cancer therapy. On the other hand, parameters of systemic inflammation can be confounding factors in a cancer biomarker study. Stricter sample stratification for biomarker studies and extensive independent validation by independent researchers may distinguish true biomarkers from the confounding factors. The results obtained by current studies seem to be promising and further validation will confirm the prognostic utility of candidate biomarkers for clinical applications (Table 1).

TREATMENT STRATEGY BASED ON THE STATUS OF PREDICTIVE BIOMARKERS

As described above, a number of molecules have emerged as predictive candidate biomarkers for the treatment of esophageal cancers and will hopefully result in establishment of biomarkers for routine clinical use. By combining several promising markers in a cross-modality manner, we may be able to develop versatile predictive tools that are more effective than single markers. This approach should be achieved by linking the biomarker components to stratified patient information. The diagnostic kit may be developed such that it gets a local makeover to adjust for variations in clinical therapeutic approaches. The effectiveness of response prediction depends on therapeutic strategies, including the surgical procedure and neoadjuvant therapy, and the clinical background of patients with esophageal cancer. For example, neoadjuvant chemotherapy with cisplatin plus 5-fluorouracil is the current standard treatment for locally advanced esophageal cancer in Japan[86], while neoadjuvant CRT with cisplatin plus 5-fluorouracil is the standard in Western countries[87]. In Japan, a three-arm Phase Ⅲ trial started in November 2012 to confirm the superiority of docetaxel and cisplatin plus 5-fluorouracil over cisplatin plus 5-fluorouracil and the superiority of cisplatin plus 5-fluorouracil with CRT over cisplatin plus 5-fluorouracil as neoadjuvant therapy for esophageal SCC[88]. If neoadjuvant chemotherapy is combined with radiation therapy, the prediction kit should include the biomarkers associated with sensitivity to radiation, such as RNA-binding protein RNPC1[89]. On the other hand, if the combination chemotherapy regimen includes docetaxel, a docetaxel-specific biomarker, such as RPN2[90], should be present. In addition, a predominant histological type of esophageal cancer has been found to exhibit region-dependent differences. Thus, SCC is the predominant histological type of esophageal carcinoma worldwide; however, in Australia, the United Kingdom, the United States, and some Western European countries (*e.g.*, Finland, France, and the Netherlands), the incidence of esophageal AC now exceeds that of SCC[91,92]. In a study on 8562 patients who underwent surgical resection, Merkow *et al*[93] found that the only factor predictive of pathological complete response was SCC histology. The response pattern to neoadjuvant therapy is different in each histological type[94]. Thus, to increase the specificity of response prediction, different molecules can serve as biomarkers depending on histological type. Any article clubbing two diseases together is not appropriate. Surgical procedures are also different in each country. Surgical options for the resection of esophageal carcinoma include the following: trans-hiatal esophagectomy and trans-thoracic approaches, such as Ivor Lewis esophagectomy (abdominal and right thoracic approach also called the Lewis-Tanner approach), the three-incision modified McKeown esophagectomy (involving laparotomy, right thoracotomy, neck anastomosis, and left thoracotomy) and the left thoraco-abdominal approach[95-101]. In Japan and several other countries, extended lymphadenectomy is a common procedure, but this is not the case elsewhere[102-104]. In conclusion, because the sensitivity and specificity of response prediction vary according to regional differences in therapeutic strategies and clinical background, it may be necessary to customize a prediction kit for each country rather than to adopt a universal prediction strategy.

Table 1 Molecular biomarkers for predicting the response to neoadjuvant therapy in esophageal cancer

Modality/biomarker	*N*	Histology	Neoadjuvant therapy	Sensitivity	Specificity	PPV	NPV	Accuracy	Ref.
Gene expression profiling									
5 genes (EPB41L3, RNPC1, RTKN, STAT5B, and NMES1)	13	Squamous-23% Adeno-77%	CRT; 5-FU and cisplatin, 40.05-44 Gy	100%	91%	NA	NA	95%	[15]
3 genes (PERP, S100A2, and SPRR3)	19	Squamous-11% Adeno-84%	CRT; 5-FU, docetaxel and irinotecan, 50.4 Gy	86%	85%	75%	92%	85%	[16]
Ephrin B3 receptor	47	Adeno-100%	CT; 5-FU, cisplatin and leucovorin	89%	84%	89%	84%	87%	[17]
199 genes	25	Squamous-100%	CT; 5-FU, cisplatin and adriamycin	68%	93%	88%	79%	82%	[18]
32 genes	46	Squamous-46% Adeno-54%	CRT; 5-FU and cisplatin, 35-50 Gy	100%	67%	55%	100%	76%	[19]
Single nucleotide polymorphisms									
XRCC1 R399Q	210	Squamous-17% Adeno-83%	CRT; 5-FU, cisplatin and paclitaxel, RT (NA)	NA	NA	NA	NA	NA	[26]
ERCC1 C118T/XRCC1 A194G	52	Squamous-60% Adeno-40%	CRT; 5-FU and cisplatin, 36 Gy	54/5%	67/100%	80/100%	37/59%	58/60%	[27]
MicroRNAs									
miR-192, miR-194	8	Squamous-25% Adeno-75%	CRT; 5-FU and cisplatin, 40 Gy	NA	NA	NA	NA	NA	[40]
HS-240, has-miR-296, has-miR-141, has-miR-31, HS-217	25	Squamous-20% Adeno-80%	CRT; cisplatin and irinotecan, 50.4 Gy	NA	NA	NA	NA	NA	[41]
Serum miR-200c	64	Squamous-100%	CT; 5-FU, cisplatin and adriamycin or docetaxel	68%	62%	53%	75%	64%	[43]
miR-200c	98	Squamous-91%	CT; 5-FU, cisplatin and adriamycin	NA	NA	NA	NA	NA	[44]
miR-31	19	Squamous-5% Adeno-95%	CRT; 5-FU and cisplatin, 40.05 Gy	NA	NA	NA	NA	NA	[45]
Proteomics									
Mitochondrial respiratory chain complexes	69	Adeno-100%	CT; 5-FU and cisplatin	50%	93%	82%	74%	71%	[53]
C4a, C3a	31	Squamous and adeno; NA	CRT; 5-FU and cisplatin, 40-44 Gy	79%	83%	NA	NA	81%	[54]
Immunohistochemistry									
CD44+/CD24-	27	Adeno-100%	CRT; NA	50%	100%	100%	50%	67%	[64]
EGFR	37	Squamous-100%	CT; 5-FU, cisplatin and docetaxel	93%	55%	58%	92%	70%	[65]
HER2/neu	34	Squamous-100%	CRT; 5-FU and cisplatin or leucovorin, 39.6-40 Gy	69%	71%	60%	79%	71%	[66]
p53 (wild-type)	1497	Squamous-91% Adeno-9%	CRT or CT (meta-analysis)	NA	NA	NA	NA	NA	[73]
MLH1, FANCD2	79	Squamous-27% Adeno-71%	CRT; 5-FU, cisplatin and/or paclitaxel, 45-64.8 Gy	20%	100%	100%	22%	35%	[77]
Heat-shock proteins and glucose-regulated proteins	90	Adeno-100%	CT; 5-FU, cisplatin or oxaliplatin	61%	63%	53%	70%	62%	[80]
Serum biomarker									
Serum soluble interleukin-6 receptor	37	Squamous-100%	CRT; 5-FU and cisplatin, 40 Gy	NA	NA	NA	NA	NA	[81]
Thymidylate synthetase and dihydropyrimidine dehydrogenase	29	Squamous-34% Adeno-66%	CRT; 5-FU and cisplatin, 36 Gy	20%	100%	100%	36%	45%	[82]
Common blood tests									
Neutrophil-to-lymphocyte ratio	83	Squamous-84%	CT; 5-FU and cisplatin	71%	66%	56%	79%	68%	[83]
Albumin	246	Squamous-13% Adeno-86%	CT; cisplatin, epirubicin and 5-FU or capecitabine, or epirubicin and oxaliplatin	NA	NA	NA	NA	NA	[84]

PPV: Positive predict value; NPV: Negative predict value; Squamous: Squamous cell carcinoma; Adeno: Adenocarcinoma; CRT: Chemoradiotherapy; CT: Chemotherapy; 5-FU: 5-fluorouracil; NA: Not available.

Pathological nonresponders to neoadjuvant therapy for esophageal cancer demonstrate no survival benefits compared to patients treated with primary esophagectomy[12]. Factors predicting the response to neoadjuvant therapy may help to reduce the number of unnecessarily treated patients and lead to the investigation of new and more effective therapeutic strategies for the unresponsive group. However, if there are no effective therapies for nonresponders, predicting the response to neoadjuvant therapy is tantamount to abandoning nonresponders to their fate. Further improvement in outcomes for the patient with esophageal cancer cannot be

achieved without improvement of the prognosis of nonresponders. Therefore, the development of new effective therapies for nonresponders concurrently with progress in predictive methodology is necessary. Recently, novel therapeutic approaches, such as new targeted strategies, epigenetic therapeutics, monoclonal antibody therapy and carbon-ion radiotherapy, are being developed[105-107]. Although initially many of these studies involved patients with metastatic disease, these therapies are now being increasingly investigated in the preoperative setting as components of multimodality therapy[105]. The efficacy of targeted agents for neoadjuvant therapy of patients with esophageal cancer has yet to be established in previous and ongoing clinical trials[106]. Additional trials to examine new targeted agents have been performed. Further improvement of the prognosis of esophageal cancer patients can be achieved through the introduction of these novel therapeutic approaches in practice, which provides prognostic improvement for nonresponders identified by predictive biomarkers.

CLINICAL APPLICATION OF BIOMARKERS

Advances in modern omics technologies and the integration of the results into clinical practice provide valuable opportunities for biomarker discovery research. As discussed in this review, considerable numbers of promising biomarkers in esophageal cancer have been established and more biomarker candidates are likely to be identified by the application of novel technologies. These biomarkers have been discovered through a hypothesis-driven approach by medical doctors for specific clinical applications and they seem to have great potential in providing benefits to patients. However, only a few of the biomarkers discovered in the last decade have been introduced into clinical practice and skepticism about the clinical utility of biomarkers in the diagnosis and treatment of cancer has been expressed[108]. As discussed here, treatments based on the results of biomarker studies should be further developed to benefit all patient subgroups. To establish the reliability of biomarkers before clinical trials, the reproducibility of the results should be assessed by independent investigators. However, we found that none of the biomarkers reviewed in this article had been validated by other researchers. Small sample sizes may be the most serious obstacle for validation of predictive biomarkers. Although it is generally accepted that multi-institutional and inter-disciplinary collaboration is required for biomarker validation, until now no serious validation studies have been performed for any predictive biomarkers in esophageal cancer and this issue requires further analysis.

CONCLUSION

We have reviewed the current status of biomarkers in esophageal cancer, especially focusing on the utility for predicting responses to neoadjuvant therapy. The reported biomarkers seem to be promising because they have been developed based on clinical research and their predictive performance has been examined by using clinical samples. Further validation and functional evaluation will increase the reliability of these biomarkers. Combined use of the reported biomarkers may increase prognostic performance and this concept is worth further research. Prognostic modalities should be tailored to specific clinical therapeutic approaches that differ according to individual cases. The development of new effective therapies for nonresponders can be hoped for with the progress in predictive techniques. Further understanding of the molecular mechanisms underlying the resistance to CRT in cancers can be achieved by investigating the functional effects of biomarkers on the malignant properties of tumor cells and such efforts will pave the way to novel therapeutic strategies.

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REVIEW

Epidemiological studies of esophageal cancer in the era of genome-wide association studies

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Abstract

Esophageal cancer (EC) caused about 395000 deaths in 2010. China has the most cases of EC and EC is the fourth leading cause of cancer death in China. Esophageal squamous cell carcinoma (ESCC) is the predominant histologic type (90%-95%), while the incidence of esophageal adenocarcinoma (EAC) remains extremely low in China. Traditional epidemiological studies have revealed that environmental carcinogens are risk factors for EC. Molecular epidemiological studies revealed that susceptibility to EC is influenced by both environmental and genetic risk factors. Of all the risk factors for EC, some are associated with the risk of ESCC and others with the risk of EAC. However, the details and mechanisms of risk factors involved in the process for EC are unclear. The advanced methods and techniques used in human genome studies bring a great opportunity for researchers to explore and identify the details of those risk factors or susceptibility genes involved in the process of EC. Human genome epidemiology is a new branch of epidemiology, which leads the epidemiology study from the molecular epidemiology era to the era of genome wide association studies (GWAS). Here we review the epidemiological studies of EC (especially ESCC) in the era of GWAS, and provide an overview of the general risk factors and those genomic variants (genes, SNPs, miRNAs, proteins) involved in the process of ESCC.

Key words: Esophageal cancer; Epidemiology; Genome wide association study; Single nucleotide polymorphism; MicroRNA

Core tip: Epidemiological study methods advance as the science and technique progress. In the era of genome wide association studies (GWAS), human genome epidemiology (HuGE) provide a great chance for epidemiologists and clinical scientists to explore the cause of disease and evaluate genomic biomarkers for diagnosis or prognosis. More and more epidemiological studies use GWAS methods to analyze genomic variants and the association with esophageal cancer. Here we review epidemiological studies of esophageal cancer in the era of GWAS, and briefly introduce the case-control study and cohort study methods in HuGE studies.

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INTRODUCTION

Esophageal cancer (EC) caused about 395000 deaths

Table 1 Major risk factors for esophageal cancer

Risk factor	Ref.
Cigarette smoking (tobacco use)	Fan *et al*[12], Oze *et al*[7]
Alcohol drinking (alcohol consumption)[1]	Oze *et al*[13], Fan *et al*[12], Islami *et al*[5]
Drinking hot tea or soup at high temperature	Wu *et al*[14]
Food mutagens	Yokokawa *et al*[15], Zhang *et al*[16]
Family history	Turati *et al*[9], Gao *et al*[17]
Nutritional deficiency	Tran *et al*[18]
Poor oral hygiene/ESCC	Dar *et al*[8]
Coffee consumption[2]	Naganuma *et al*[11]
HPV infection	Li *et al*[19], Cui *et al*[20]
Obesity	Chen *et al*[21]

[1]Alcohol consumption depends on the quantity of alcohol intake; [2]Coffee consumption: reverse relation. ESCC: Esophageal squamous cell carcinoma.

in 2010[1]. The incidence rate and mortality rate varied among different geographic and ethnic populations. China has the most cases of esophageal cancer. EC is the fourth leading cause of cancer associated death in China. Esophageal squamous cell carcinoma (ESCC) is the predominant histologic type (90%-95%), while the incidence of esophageal adenocarcinoma (EAC) remains extremely low in China.

Traditional epidemiological studies have identified that environmental carcinogens play a critical role in the process of EC. Molecular epidemiological studies revealed that susceptibility to EC is associated with both genomic and non-genomic factors and the interaction between genomic and non-genomic factors. Of all the factors, some are associated with ESCC and others with EAC. Human genome epidemiology (HuGE) is denoted as "an emerging field of inquiry that uses systematic applications of epidemiologic methods and approaches in population-based studies of the impact of human genetic variation on health and disease"[2]. HuGE emerged after the sequencing of human genome was accomplished[3,4]. The characteristic of HuGE is the techniques applied in studies, especially the technique of DNA microarray chips used in genome-wide association studies (GWAS). These techniques can compare millions of SNPs between genome DNA from cases and controls. In this review we focus on the epidemiological studies of EC in the era of GWAS.

GENERAL RISK FACTORS FOR EC

The incidence of EC is associated with age. More than 85% of EC patients were diagnosed at an age more than 55 years old. The incidence of EC in males is higher than that in females. Esophageal reflux disease (GERD) is a risk factor of EAC. GERD is also a risk factor for Barrett's esophagus (BE), and BE is associated with an increased risk for EC. Asian, especially Chinese, are more like to have an onset of EC than other populations.

Tobacco use (tobacco smoking, tobacco chewing, *etc.*) is a predominant risk factor for EC, especially ESCC. Alcohol drinking can also increase the risk of EC. Alcohol drinking is more likely to increase the risk of ESCC. People exposed to both tobacco use and alcohol had the risk of EC much more than those exposed to smoking or drinking alone. The risk of ESCC increased as the quantity of alcohol intake increased. The association between alcohol drinking and an increased risk of EC was more likely observed in Asian populations than in others[5]. Alcohol consumption and cigarette smoking are risk factors for ESCC in China and Japan[6,7].

Overweight or obesity is associated with a higher risk of EAC. A diet with more fruits or/and vegetables is reported to reduce the risk of EC. On the contrary some diet habit may raise the risk for EC. Drinking very hot liquids frequently may increase the risk of ESCC. Overeating is the risk factor for EAC.

Infection with human papillomavirus (HPV) is associated with a number of cancers. HPV infection has been observed in about one-third of EC patients in Asia and South Africa.

Risk factors for EC varied among different countries, which may explain in part by the social-economic difference. The risk factors for EC are different between high- and low-incidence areas[6]. A study in Kashmir[8] recruited 703 cases and 1664 controls and found an inverse association between tooth cleaning and ESCC risk. A study based on a network of Italian and Swiss case-control studies found that a family history of oral and pharyngeal cancer was associated with an increased risk for EC[9]. In China individuals with a family history of EC were found to have an increased risk for EC[10]. The Miyagi Cohort Study found that people who drink one or more cups of coffee per day compared with those who did not drink have a lower risk of EC and oral pharyngeal cancer[11]. The major risk factors for EC are summarized in Table 1.

GENERAL VIEW OF EPIDEMIOLOGY IN THE EAR OF GWAS

Epidemiology studies in the era of GWAS are characterized by large sample size and the use of the technique of microarray. HuGE has advanced to the stage of GWAS[22-26]. Table 2 shows the genomic variants identified to be associated with ESCC. Some of those genetic variants were confirmed in other populations and some others were not identified in other populations. GWAS in China showed that variants in several chromosome regions conferred an increased risk of EC, but only genetic variants in alcohol-metabolizing genes were risk factors for ESCC in Japanese[6,22-26]. A 2-step GWAS including 1070 cases and 2836 controls identified that single nucleotide polymorphisms (SNPs) rs671, rs1229984, alcohol consumption, and tobacco use were risk factors for ESCC[23].

Genetic polymorphisms can affect the susceptibility

Table 2 Genomic variants identified to be associated with esophageal squamous cell carcinoma

Loci associated with ESCC	Method/design	Case sample size	Control sample size	Ref.
PLCE1 (10q23 rs227422) and C20orf54 (20p13)	GWAS	1077	1733	Wang *et al*[22]
ALDH2 (4q21-23, rs671) and ADH1B (12q24, rs1229984)	GWAS	1070	2836	Cui *et al*[23]
PLCE1 (10q23 rs2274223)	GWAS	2115	3302	Abnet *et al*[24]
ALDH2 (4q23, rs671) and ADH1B (12q24.11–13, rs1229984)	GWAS	1071	2762	Tanaka *et al*[25]
5q11 (rs10052657) 21q22 (rs2014300), 6p21 (rs10484761), 10q23 (rs2274223), and 12q24 (rs2074356, rs11066280)	GWAS	2031	2044	Wu *et al*[26]
CYP1A1 A2455G polymorphism(Ile/Val, rs1048943)	Meta-analysis (13 case-control studies)	1881	3786	Shen *et al*[27]
CYP1A1/CYP2E1	Case-control study	565 /482	468/466	Wang *et al*[28]
(MTHFR) C677T and A1298C polymorphisms with ESCC	Meta-analysis (15 case-control studies)	3213	4354	Fang *et al*[29]
rs1014867 polymorphisms in FAT4 gene	Case-control study	2139	2273	Du *et al*[30]
Interleukin 1B rs16944	Case-control study	380	380	Zheng *et al*[31]
CHRNA5-A3-B4 rs667282 TT/TG	Case-control study	866	952	Wang *et al*[32]
rs1494961, rs1229984 and rs1789924, and rs671	Case-control study	2139	2273	Gao *et al*[33]
Genetic variants in DNA repair pathway genes/(EGFR) signaling pathway	Case-control study	1942	2111	Li *et al*[34], Li *et al*[35]
Sex hormone metabolic genes	Case-control study	1026	1452	Hyland *et al*[36]
Chromosome 1 open reading frame 10 (C1orf10)	Case-control study	991	984	Zhang *et al*[37]

ESCC: Esophageal squamous cell carcinoma; GWAS: Genome wide association studies; PLCE1: Phospholipase C epsilon 1; C20orf54: Chromosome 20 open reading frame 54; ADH1B: Alcohol dehydrogenase; ALDH2: Acetaldehyde dehydrogenase.

to EC. Cytochrome P450 1A1 (CYP1A1) enzyme is a member of the CYP superfamily and prone to mutation, and an association between CYP1A1 enzyme activity and the risk of developing EC was revealed[38]. A meta-analysis uncovered that the A2455G polymorphism (Ile/Val, rs1048943) was a risk factor for EC[27]. By combining the technique of DNA microarray and epidemiology data of EC patients living in North or South China, the polymorphisms of CYP1A1 and CYP2E1 were studied[28]. In South area there was a significant association between CYP1A1 m2 polymorphism and EC. In North area there were significant associations between CYP2E1 Pst I and CYP2E Rsa I polymorphisms and EC. A significantly increased risk of ESCC was identified for smokers with the methylenetetrahydrofolate reductase (MTHFR) 677T allele[29]. MTHFR 677T and MTHFR 1298C conferred an increased risk for ESCC in Chinese population than in other populations. Four SNPs (rs1014867, rs12508222, rs1039808 and rs1567047) in FAT4 as potential risk factors for EC were studied[30]. The T allele of rs1014867 (Pro4972Ser) was associated with a reduced risk for EC[30]. The functional IL1B rs16944G > A polymorphism might be associated with the risk of ESCC and IL3 rs2073506 G > A polymorphism was a risk factor for ESCC with higher TNM stages[31]. CHRNA5-A3-B4 rs667282 TT/TG genotypes were risk factors of ESCC in Chinese[32]. In China, a case-control study including 2139 cases and 2,273 controls was carried out to evaluate the associations of six reported SNPs (rs1494961, rs1229984, rs1789924, rs971074, rs671 and rs4767364) with risk of ESCC. Results indicate that rs1494961, rs1229984, rs1789924, and rs671 may be used as biomarkers for ESCC[33]. Based on the SNPs identified in GWAS, 25 SNPs, 4 non-genomic factors (sex, age, tobacco use and alcohol drinking) and their associations with ESCC risk were studied[39]. Results indicate that genomic factors, none-genomic factors and their interactions can predict who are at high risk for ESCC. In contrast to association with a risk of ESCC in Asians, the PLCE1 rs2274223 and RFT2 13042395 SNPs were not associated with a risk of EC in Dutch Caucasians[40]. GWAS also identified three SNPs (rs10419226 in CRTC1, rs11789015 in BARX1 and rs2687201 near FOXP1) that were associated with a risk of EAC and BE[41].

GENOMIC VARIANTS IN PATHWAY GENES AND THEIR ASSOCIATIONS WITH EC

A GWAS aimed to explore the DNA repair pathway genes as risk factors for ESCC and GC was carried out[34]. One thousand six hundred and seventy-five SNPs were genotyped in cases (ESCC, GC) and controls from Shanxi and Linxian[34]. The DNA repair pathway genes were found to be risk factors for ESCC. CHEK2 was significantly associated with ESCC. Li *et al*[35] explored 3443 SNPs in genes involved in the EGFR signaling pathway in a study including 1942 ESCC cases, 1758 GC cases, and 2111 controls. Gene-level analyses found that GNAI3, CHRNE, PAK4, WASL, and ITCH were associated with a risk of ESCC[35]. A study analyzed 797 SNPs in 51 sex hormone metabolic genes in 1026 cases and 1452 controls[36]. Six genes including SULT2B1, CYP1B1, CYP3A7, CYP3A5, SHBG and CYP11A1 were identified as risk factors for ESCC[36]. Chromosome 1 open reading frame 10 (C1orf10), which is involved in heat shock and ethanol response, is either absent or down-regulated in ESCC tissues. Six strongly linked SNPs in a region of 7 kb were observed in a case-control study[37]. Compared

Table 3 MicroRNA expression and their associations with esophageal squamous cell carcinoma[45]

MiRNA	Compared to normal esophageal tissue	Proved targets
miR-10a	Decreased	HOXA3, HOXB1, HOXB3 HOXD4, HOXD10
miR-21	Increased	PCDCD4, NFIB, PTEN, TPM1
miR-93	Increased	FUSA, E2F1, TP53, INP1
miR-129	Increased	LATS2
miR-203	Increased/ decreased	ABL1, TP53INP1, SOCS3
miR-205	Decreased/increased	ZEB1, ZEB2, E2F5, HER3, ERBB3, PRKCE, LRP1
miR-375	Decreased	PDK1

with -1139GG, -1139CC genotype was a risk factor for ESCC[37].

The HuGE progressed form the discovery of novel genes or SNPs to the functional or mechanistic study of those genes or SNPs. Moreover, HuGE studies try to screen some of those genes, SNPs or miRNAs that are clinical treatment targets or biomarkers for diagnosis or prognosis. A low mtDNA copy number variant (CNV) was a risk factor for EAC[42]. A case-control study was carried out to analyze the relationship between SNPs (rs17417407, rs2274223 and rs22744224) in PLCE1 and susceptibility to ESCC[43]. Rs2274223G was identified to be a risk factor for ESCC, and rs2274224G was observed as a favorable factor for ESCC[43]. Phenotypes for rs17417407T, rs2274223G and rs2274224G were observed as risk factors for ESCC. Genomic polymorphisms in PLCE1 can affect the risk of ESCC in Chinese population exposed to tobacco smoking[43].

Zhang *et al*[37] found that there was an interaction between the -1139G/C genotype in C1orf10 and smoking, which increases the risk of ESCC. An HPV gene chip was used to detect HPV genotypes in 183 EC cases and 89 controls[20]. The frequency of seven HPV genotypes (16, 18, 35, 52, 6, 11, 43) in EC tissues was higher (31.7%) than that in controls (9.0%, $P < 0.001$), indicating that HPV infection was a risk factor for EC in Kazakh population. Moreover, heterozygote rs2274223 in PLCE1 was associated with an increased risk of HPV infection[20].

MICRORNAS AND THEIR ASSOCIATIONS WITH EC

MicroRNAs (miRNAs) are non-coding RNAs that modulate the translation of RNAs. MiRNAs have been involved in cancer initiation and development. Different miRNAs show differential expression levels in EC tissue or EC cell lines. The levels of miR-145 and miR-143 were decreased in ESCC tissues. An inverse association between miR-143 expression levels and cancer invasion or metastasis was identified[44]. Results showed that miR-143 may act as a suppressor in the process of ESCC. MiRNA microarray technique can be used to explore the profiles of miRNAs in ESCC cell lines. MiR-10a and MiR-205 were observed as potential specific biomarkers for ESCC (Table 3)[45].

Kan and Meltzer[46] reviewed miRNAs in BE and EAC. They surmised the following: (1) miRNA profiles were different between BE and EAC; (2) miR-196a is overexpressed in EAC tissues and is favorable to EAC cell survival; miR-196a might be a biomarker during the carcinogenesis from BE to EAC; and (3) the miR-106b-25 polycistron is involved in EC progression via suppression of p21 and Bim. The potential role of miRNAs in GC and EC and the mechanisms of action have been reviewed previously[47].

MiRNAs participate in the process of carcinogenesis by affecting the expression of genes to regulate cell apoptosis, proliferation and invasion. Some miRNAs have been proved to be associated with the characteristics of cancer or the survival time of patients, and those miRNAs might be valuable as biomarkers for diagnosis or prognosis prediction. A greater understanding of functions of miRNAs in EC could provide more details about the mechanisms of carcinogenesis (Table 4)[44,47,48].

A study explored the expression of miRNAs in ESCC and found that 15 miRNAs were down-regulated[48]. Four miRNAs (miR-145, miR-30a-3p, miR-133a and miR-133b) were decreased in ESCC and might act as tumor suppressors. Three miRNAs (miR-133b, miR-133a and miR-145) can directly inhibit FSCN1 expression, which might decrease the risk for ESCC[48]. A hospital based case-control study including 380 cases and 380 controls was carried out to observe the association of SNPs in miRNAs with genetic susceptibility to ESCC[49]. Female individuals or people who never smoke or drink have a lower risk for ESCC if they carry MiR-196a2 rs11614913 T > C[49]. Zhang *et al*[50] reported that up-regulation of miR-203 in EC cells can significantly increase apoptosis and decrease miR-21 expression. MiR-203 overexpression can also inhibit cell invasion, migration and proliferation, and may act as a tumor suppressor in EC.

CLINICAL RESEARCH OF GENOMIC BIOMARKERS FOR EC

EC is a disease with a poor prognosis[51]. It is urgent to identify valuable biomarkers involved in the diagnosis, progress or therapy targets for ESCC. Qi[52] reviewed the proteins, identified by proteomics, which were associated with the process of ESCC. The mechanisms of action of the proteins identified by proteomics and involved in the progress of ESCC were also discussed[53].

Loss of chromosome 19p is one of the most frequent allelic imbalances in ESCC. Down-regulation of DIRAS1 was associated with a poor survival rate. About 50% of ESCC cases had down-regulation of DIRAS1, and this down-regulation was associated with unfavorable clinical characteristics such as lymph node metastasis and low survival rate[53]. A GWAS observed the relationship between SNPs and the survival of ESCC

Table 4 Common miRNA expression profiles in esophageal cancer[47]

ESCC		EAC	
Up-regulated	Down-regulated	Up-regulated	Down-regulated
miR-21	Let-7c	miR-21	Let-7c
miR-155	miR-1	miR-28	miR-203
miR-93	MiR-99a	miR-3a-5p	miR-205
miR-129	miR-100	miR-143-145 cluster	miR-23a
	miR-133a	miR-192	miR-27a
	miR-143-145 cluster	miR-194	miR-27b
	miR-203	miR-215	miR-31
	miR-375		miR-99a
			miR-100

ESCC: Esophageal squamous cell carcinoma; EAC: Esophageal adenocarcinoma.

patients[54]. Results showed that SLC39A6 overexpression was associated with a shorter period of survival, which indicated that SLC39A6 might be a target for ESCC therapy[54]. HOTAIR, a well-known long non-coding RNA, has been reported to associate with ESCC. It was found that HOTAIR was overexpressed in ESCC compared to normal esophageal tissues[55,56]. Overexpression of HOTAIR was associated with poorer prognosis. The HOTAIR /WIF-1 axis was identified to play an important role in cell metastasis and might be a target for ESCC therapy. PIK3CA mutations in ESCC are associated with longer survival, suggesting its role as a prognostic biomarker[57]. Proteomic methods were used to evaluate proteins as potential biomarkers for ESCC[58], and 33 proteins overexpressed and 14 proteins down-regulated in ESCC were identified[58]. The expression of fos related antigen 1 (Fra-1) was identified as an unfavorable factor for prognosis[59]. The effect of SNPs of long intergenic non-coding RNAs on ESCC was studied by Wu *et al*[60]. 52 SNPs were studied in 1493 ESCC cases and 1553 controls in China[60]. Compared with the AA genotype of rs11752942, AG and GG reduced the risk of ESCC. Rs11752942G allele could significantly down-regulate the expression level of lincRNA-uc003opf.1[60]. These results indicated that rs11752942 in lincRNAuc003opf. 1 exon was a biomarker for susceptibility to ESCC. Sakai *et al*[61] reviewed the most recent studies on miRNAs in EC and/or BE. Four miRNAs were identified as diagnostic biomarkers and five miRNAs were supposed to be valuable biomarkers for diagnosis and prognosis. The progress in miRNAs identified in EC is exciting, but there is still a lot of work to be done before those miRNAs can be used as biomarkers for diagnosis, efficacy evaluation or prognosis prediction.

EPIDEMIOLOGICAL STUDY DESIGN IN THE ERA OF GWAS

The advantages and disadvantages of case-control and cohort studies in the era of GWAS have been previously discussed in detail[62]. The great majority of GWAS conducted to date have used the case-control design, in which genome or SNPs were compared between tissues from esophageal cancer patients or esophageal cancer free controls[22,26]. Other risk factors for EC were also investigated and analyzed to search for the genetic and environmental factors influencing EC. Case-control design not only allows to study multiple factors that might associate with disease, but also permits a more detailed evaluation of risk factor exposure, such as tobacco use, alcohol drinking, occupational, HPV infection, family history of EC or dietary history. However, there are several biases that are related with the selection of cases and controls. If cases can be representative of all persons who develop EC, the bias from case selection in a case-control study is limited. However, cases in most of the case-control studies are often hospital based, typically through review of medical records, and those with early death have great chance not to be included, leading to survival bias. Theoretically, controls should be representative of all persons at risk for EC. In fact, selecting controls in a case-control study is the most difficult aspect. The evaluation of risk factor exposure should avoid bias, which is related to measuring exposures. Case-control studies are often easier and cheaper to conduct than cohort studies.

The major merit of the cohort study is that recall bias is controlled by collecting exposure prior to disease outcome. Cases identified in cohort are incident and free of survival bias. Results of cohort studies can be used to explain the cause of disease. The disadvantages of cohort studies include the requirement of large sample size if the incidence of disease is low, expensive cost for genomic test, and long term follow-up[63]. Due to reasons of cost and efficiency fewer GWAS use cohort study design. More and more case-control studies were carried out with large sample sizes, to explore the genomic and environmental risk factors for EC[23,25].

GWAS use high-throughput microarray technologies to analyze genetic SNPs, miRNAs or proteins and evaluate their association with disease or with clinical utilities (biomarkers for diagnosis or prognosis). Since 2005, more than 100 loci for more than 40 diseases have been discovered and confirmed. Many SNPs were first observed to be associated with disease risk. GWAS have some advantages in identifying genetic variants associated with disease. GWAS also have some limitations, including type I and type II errors and biases due to poor representative of participants. Two step or multi-step GWAS are recommended in epidemiological case-control studies.

CONCLUSION

The flood of GWAS findings from case-control studies has led to the increasing need for subsequent confirmation and functional studies in experimental systems to identify the biological mechanisms of the association be-

tween genomic variants and EC. Epidemiological studies of EC in the era of GWAS have explored the genomic variants affecting signaling, epigenetic regulation, RNAs, proteins and pathways involved in cell proliferation or invasion. However, much work remains to be done including identifying the biomarkers for screening, efficacy evaluation and prognosis prediction. In the future, more and more epidemiological studies will take the advantages of population-based, very large sample-sized GWAS.

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REVIEW

Perihilar cholangiocarcinoma: Current therapy

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Abstract

Perihilar cholangiocarcinoma, which is a rare primary malignancy, originates from the epithelial cells of the bile duct. Usually invading the periductal tissues and the lymph nodes, perihilar cholangiocarcinoma is commonly diagnosed in the advanced stage of the disease and has a dismal prognosis. Currently, complete hepatectomy is the primary therapy for curing this disease. Perioperative assessment and available surgical procedures can be considered for achieving a negative margin resection, which is associated with long-term survival and better quality of life. For patients with unresectable cholangiocarcinoma, several palliative treatments have been demonstrated to produce a better outcome; and liver transplantation for selected patients with perihilar cholangiocarcinoma is promising and desirable. However, the role of palliative treatments and liver transplantation was controversial and requires more evidence and substantial validity from multiple institutions. In this article, we summarize the data from multiple institutions and discuss the resectability, mortality, morbidity and outcome with different approaches.

Key words: Cholangiocarcinoma; Klatskin tumor; Surgery; Liver transplantation; Therapy

Core tip: Perihilar cholangiocarcinoma is a type of malignant tumor with vague and insidious symptoms, and is often diagnosed at an advanced stage. Currently, negative margin resection (R0) is the only way to cure patients with perihilar cholangiocarcinoma. In this article, we describe the surgical procedure and the criteria for operation and illustrate the palliative therapy and liver transplantation options for unresectable perihilar cholangiocarcinoma.

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INTRODUCTION

Cholangiocarcinoma, which is a rare malignant tumor, constitutes less than 1% of all human malignancies[1]. The spectrum of cholangiocarcinoma is divided into three types, according to the anatomical location. Perihilar cholangiocarcinoma (PHC) is the most common type of the malignant tumor accounting for 50%-67% of all cases, followed by distal cholangiocarcinoma (DCCA) and intrahepatic cholangiocarcinoma (ICCA), which account for 27%-42% and 6%-8%, respectively[2,3]. When first described by Klatskin, PHC was commonly called Klatskin tumor[4]. Ben-Menachem summarized that the most common risk factors of PHC were liver flukes, primary sclerosing cholangitis, choledochal cysts, hepatolithiasis and cirrhosis, which account for 10% of the cases[5]. Patients with PHC are usually admitted to the hospital with severe painless jaundice and are diagnosed at an advanced stage, which means a poor prognosis and a shortened life span.

Complete resection is recognized as an effective therapy for many carcinomas. Similarly, resection has long

been demonstrated to be the best option for patients with PHC, and is associated with long-term survival and better quality of life[6]. PHC surgery was previously considered to be a challenge for hepatobiliary surgeons, because of the complex, intimate and variable anatomical relationship of the bile duct and vascular structures[7]. Because of the anatomical characters and the slow progression of the tumor, palliative procedures have been used to treat cancers involving the hepatic hilus, whereas definitive surgery can only be applied to a minority of patients with well-localized lesions[8]. From 1955 to 1973, Longmire collected 63 patients with extrahepatic cholangiocarcinoma (ECCA), and 34 of those patients had lesions that originated near the confluence of the hepatic duct. However, only six patients (18%) were likely candidates for hepatic resection. Guthrie *et al*[9] gathered 107 patients with ECCA divided into two periods, 1980-1985 and 1986-1991. They found that the overall resectablity rate (17%) was similar to that reported in other studies, while the use of percutaneous transhepatic cholangiography decreased and the use of endoscopic retrograde cholangiography increased in the second period. However, palliative treatments had unsatisfactory results and were associated with a high incidence of recurrent cholangitis and jaundice. Furthermore, the palliative approaches did not provide a method for curing the tumors; the techniques only served to relieve the symptoms of biliary obstruction.

With the development of radiology, oncology, liver transplantation and a better understanding of the pathways of tumor spread, surgical methods have recently improved significantly. Radical resection with a microscopically negative margin is believed to be the only way to cure patients with PHC. During recent decades, various surgical innovations and strategies have been introduced to achieve this goal. Currently, left or right hepatic resection, routine caudate lobe resection, lymphadenectomy, vascular resection and portal vein arterialization were promoted to improve outcome in patients with PHC. Nevertheless, for those patients who were not candidates for curative resection, several palliative treatments, such as chemotherapy, radiotherapy and photodynamic therapy, could be used to improve the quality of their life.

SURGERY

Staging and assessment of resectability

For various types of cancers, the American Joint Committee on Cancer (AJCC) TNM staging system is the most useful classification. The latest AJCC edition (7th edition) separates the ECCA into PHC and DCCA, which shows that the two subtypes have their own characteristics in pathology, treatment and prognosis. Based on the primary tumor (T), regional lymph nodes (N) and metastasis (M), the stage group is divided into 0-Ⅳ. Except for the "basic stage", the TNM classification has additional descriptions for residual tumor and histological grade. This classification is usually associated with the histological classification, also known as pathological staging, which is mostly used to stage tumors after surgical resection[10]. However, the majority of experts thought that the classification failed to indicate local respectability of the tumor and to distinguish between various surgical options, which limited the use of the staging system in the preoperative setting[11].

Proposed in the 1970s, the Bismuth-Corlette classification is the most useful stage system for predicting the resectability and for assessing the longitudinal intraductal extension of resection. Four types are classified according to the location and the longitudinal extension of the tumor in the biliary tree. Type Ⅰ lesions involve the common hepatic duct immediately below the confluence; Type Ⅱ lesions involve the hepatic bile duct confluence, that is beyond the confluence; Type Ⅲa and Ⅲb lesions occlude the common hepatic duct and either the right or the left hepatic duct, respectively; and Type Ⅳ lesions involve the confluence and both right and left hepatic ducts[12,13]. In Bismuth's opinion, Types Ⅰ and Ⅱ lesions would require only a local resection of the bile duct with a hepaticojejunostomy reconstruction, whereas the right or left hepatectomy for Type Ⅲa or Ⅲb lesions and hepatectomy plus liver transplantation for Type Ⅳ lesions, could be a contraindication for resection[13]. However, the Bismuth classification fails to describe the radical extension of the cancerous lesion and cannot provide complete information concerning vascular involvement and lymph node involvement, distant metastasis and liver atrophy. Thus, the staging system is primarily used as a convenient guideline for a surgical approach.

Combining the radial and longitudinal extensions of PHC, a preoperative clinical staging system was introduced by Jarnagin and Blumgart at Memorial Sloan-Kettering Cancer Center (MSKCC). This system, which was formally summarized and published in 2001, is also known as the T-staging system , and consists of local tumor extent, biliary duct, portal vein and hepatic lobar atrophy (Table 1)[14,15]. This system could be used to stratify patients preoperatively for the likelihood of respectability and to counsel patients on the potential for an R0 resection. In 2007, Chen *et al*[16] used this staging system to assess 85 patients with PHC. The 1-year survival rates of T1, T2 and T3 patients were 71.8%, 50.8% and 12.9%, respectively; whereas the 3-year survival rates were 34.4%, 18.2% and 0%, respectively[16]. The patients with PHC in the T1 and T2 stages were likely candidates for curative resection, whereas those in the T3 stage could not achieve R0 resection even if they had undergone resection[16]. Another retrospective test in 380 patients showed that the R0 resection rates for T1, T2 and T3 patients were 44.1%, 36.1% and 1.3%, respectively; whereas the median survival was 22.8, 23 and 10.8 mo, respectively[17]. Both surveys demonstrated that the T stage was associated with resectability and long-term survival. Moreover, the MSKCC provided the criteria for unresectable PHC, which included the following: locally

Table 1 Memorial sloan-kettering cancer center classification

Stage	Criteria
T1	Tumor involving biliary confluence ± unilateral extension to second-order biliary radicles.
T2	Tumor involving biliary confluence ± unilateral extension to second-order biliary radicles and ipsilateral portal vein involvement ± ipsilateral hepatic lobar atrophy
T3	Tumor involving biliary confluence + bilateral extension to second-order biliary radicles; or unilateral extension to second-order biliary radicles with contralateral portal vein involvement; or unilateral extension to second-order biliary radicles with contralateral hepatic lobar atrophy; or main or bilateral portal venous involvement

Table 2 Criteria for unresectability[15]

Patient factors
Medically unfit or otherwise unable to tolerate a major operation
Hepatic cirrhosis
Local tumor-related factors
Tumor extension to secondary biliary radicles bilaterally
Encasement or occlusion of the main portal vein proximal to its bifurcation
Atrophy of one hepatic lobe with contralateral portal vein branch encasement or occlusion
Atrophy of one hepatic lobe with contralateral tumor extension to secondary biliary radicles
Unilateral tumor extension to secondary biliary radicles with contralateral portal vein branch
encasement or occlusion
Metastatic disease
Histologically proven metastases to N2 lymph nodes[1]
Lung, liver, or peritoneal metastases

[1]Metastatic disease to peripancreatic, periduodenal, celiac, superior mesenteric, or posterior pancreaticoduodenal lymph nodes was considered to represent disease not amenable to a potentially curative resection. By contrast, metastatic disease to cystic duct, pericholedochal, hilar, or portal lymph nodes (*i.e.*, within the hepatoduodenal ligament) did not necessarily constitute unresectability.

advanced tumor extending bilaterally to the secondary biliary radicles, unilateral sectional bile ducts with contralateral portal vein branch involvement, encasement or occlusion of the primary portal vein proximal to its bifurcation, and atrophy of one hepatic lobe with contralateral tumor extension to sectional bile ducts (Table 2)[15].

A recent report indicated that a new system was designed by the international cholangiocarcinoma group, which incorporated the size of the tumor, the extent of the disease in the biliary system, the involvement of the hepatic artery and portal vein, the involvement of lymph nodes, distant metastases, and the volume of the putative remnant liver after resection[10]. Despite its comprehensiveness, this new classification must be validated and accepted.

We searched the key words "hilar cholangiocarcinoma", "Klatskin tumor" and "resection" using Pubmed and Medline, and we summarized the respectability and the outcomes from different institutions in different periods. The results of the surgical treatment are shown in Table 3[2,3,8,15,17-51]. Although the data were not fully calculated and were derived from tertiary referral centers, the number of patients with PHC who had undergone the resection was small, and only few large institutions contained more than 300 cases[42,50,51]. These findings attested to the rarity of this disease; additionally, these results indicated that the majority of patients lost the opportunity to undergo a curative operation when diagnosed, and therefore, these patients were not counted in the total number of study participants. Table 3 shows that the resectability rate was significantly variable, ranging from 28% to 95%, and that the curative resection rate ranged from 14% to 95%. This wide variability may be attributed to the differences in the sample content, the broad range of dates for inclusion, the characteristics of patients in different geographical areas, the methods of patient selection and the preoperative techniques in these studies.

Surgical procedures and strategies

In several reports, the surgical procedures were as follows: (1) preoperative biliary drainage was conducted to reduce the serum bilirubin concentration below 2 mg/dL; (2) preoperative percutaneous transhepatic portal embolization was performed when the volume of the liver remnant was estimated to be less than 40%; (3) the operative procedures for hilar resection were determined and planned using multidetector row computed tomography (MRCT); (4) the skeletonization of the portal vein and hepatic artery was performed using nodal clearance around the head of the pancreas; (5) portal vein resection and reconstruction were conducted before hepatic dissection if necessary; (6) frozen sections of the resected margins of the bile duct were investigated; and (7) lymph nodes in the hepatoduodenal ligament, around the head of pancreas and around the common hepatic artery were completely removed, whereas lymph nodes in the para-aortic region were removed, if possible, with a curative resection[44]. In other institutions, the surgical procedure included the hepatic artery resection, reconstruction and arterioportal shunt.

Obstructive jaundice, which is the most common symptom in patients with PHC, may increase the in-hospital mortality by 10% and is associated with many complications, such as bacterial translocation, malnutrition, renal insufficiency and postoperative liver dysfunction[52,53]. To avoid the risk of hepatic resection, preoperative biliary drainage (PBD) is recommended by many surgical teams. Percutaneous transhepatic biliary drainage (PTBD) had previously been widely used; however, several prospective randomized studies showed

Table 3 Results of surgical resection for perihilar cholangiocarcinoma

Ref.	Published year	Resections	Resectablity (%)	Negative margin (%)	Liver resection (%)	Morbidity	Mortality	5-yr survival rate (%)
Hadjis *et al*[18]	1990	27	NA	56	60	NA	7	22
Nakeeb *et al*[2]	1996	109	56	26	14	47	4	11
Su *et al*[19]	1996	49	28	49	57	47	10	15
Klempnauer *et al*[20]	1997	151	45	77	77	NA	10	28
Miyazaki *et al*[21]	1998	76	NA	71	86	34	13	26
Neuhaus *et al*[22]	1999	80	NA	61	85	55	8	22
Kosuge *et al*[8]	1999	65	73	52	80	37	9	33
Gerhards *et al*[23]	2000	112	NA	14	29	65	18	NA
Nimura *et al*[24]	2000	142	80	61	90	49	9	26
Todoroki *et al*[25]	2000	101	89	14	58	14	4	28
Jarnagin *et al*[15]	2001	80	50	78	78	64	10	26
Kawarada *et al*[27]	2002	65	89	64	75	28	2.3	26
Capussotti *et al*[26]	2002	36	NA	89	83	47	3	27
Kawasaki *et al*[28]	2003	79	75	68	87	14	1.3	22
Seyama *et al*[29]	2003	87	94	64	67	43	0	40
Rea *et al*[32]	2004	46	NA	80	100	52	9	26
Kondo *et al*[31]	2004	40	95	95	65	48	0	NA
I.Jitsma *et al*[30]	2004	42	NA	65	100	76	12	19
Hemming *et al*[33]	2005	53	50	80	98	40	9	35
Jarnagin *et al*[34]	2005	106	70	77	82	62	8	NA
Dinant *et al*[35]	2006	99	NA	31	38	66	15	27
DeOliveira *et al*[3]	2007	173	62	19	20	61	5	10
Ito *et al*[36]	2008	38	55	63	53	32	0	33
Konstadoulakis *et al*[37]	2008	59	81	68.6	86.4	25.5	6.8	34.9
Igami *et al*[41]	2010	298	70	74	98	43	2	42
Hirano *et al*[40]	2010	146	NA	87	87	44	3.4	35.5
Lee *et al*[42]	2010	302	86	70.9	89	43	1.7	32.5
Unno *et al*[44]	2010	125	NA	63.2	100	48.7	8	34.7
Ercolani *et al*[38]	2010	51	49.6	72.5	98	51	10	34.1
Shimizu *et al*[43]	2010	224	NA	69.1	78	47.6	10.7	30.3
Giuliante *et al*[39]	2010	43	29	77	93	52.5	6.9	36.1
Regimbeau *et al*[45]	2011	56	NA	76.9	100	72	8	NA
Young *et al*[48]	2012	83	92	42.2	93	62.7	7	20
Saxena *et al*[47]	2012	54	64	64.3	42	45.2	2.4	24
Ribero *et al*[46]	2012	82	NA	81.7	91.5	64.6	9.7	28
De Jong *et al*[50]	2012	305	NA	64.2	73	NA	10.6	20.2
Matsuo *et al*[17]	2012	157	78	76	90	59.2	7.6	37.5
Cheng *et al*[49]	2012	176	34	78.4	97	26.3	2.9	13.5
Nagino *et al*[51]	2013	574	76.1	76.5	96.7	57.3	4.7	32.5

NA: Not applicable.

that PTBD had no benefit in postoperative morbidity and mortality but increased potential risks, such as vascular injury, infectious complications and tumor seeding metastasis[54-56]. Currently, endoscopic nasobiliary drainage (ENBD) is performed instead of PTBD because of fewer complications and better outcomes. More recently, the Nagoya Institute demonstrated that unilateral ENBD of the future remnant lobe(s) exhibited a high success rate as an effective and suitable PBD method even in BC type Ⅲ to Ⅳ lesions[57]. To avoid the postoperative liver dysfunction resulting from extended hepatic resection, many institutions have promoted portal vein embolization (PVE) to increase volume of the future liver remnant (FLR). In several cautious surgical centers, when the FLR was 40% or less of the total liver volume, PVE was performed because the serum bilirubin level had decreased to less than 10 mg/dL[41,46]. Subsequently, surgery was performed after 2-4 wk of liver hypertrophy due to clonal expansion and cellular response[58].

When determining the surgical approach, the local excision, hepatectomy, and extended hepatectomy with or without caudate resection should be considered. In the Bismuth's opinion, Bismuth Type Ⅰ and Ⅱ would require only a local resection. Recently, bile duct resection alone without hepatectomy has been largely abandoned in favor of a more aggressive approach. Capussotti *et al*[59] conducted a systematic review of the effect of local resection compared with hepatectomy. In the pathologic aspect, the isolated bile duct cannot be adequately resected, because of the following: the necessity for wide surgical margins; neoplastic extension along the perineural sheaths and segment 1 neoplastic invasion. From another perspective, the R0 resection rate was higher after combined liver resection, although, in the earlier years of its application, local resection could be associated with fewer complications and shorter lengths of hospital stay[15,21,35]. In conclusion, according to this systematic review, local resection should only be scheduled for small papillary Klatskin tumors without bile duct confluence involvement confined to the bile duct wall[59]. Because

of the rarity and the advanced stage of the disease at the time of diagnosis, a local resection was rarely performed.

Despite the incomplete accuracy, the Bismuth classification initiated the idea of wider resection for PHC[13]. Table 3 shows that liver resection rates increased from 14% to 100% with an increased R0 resection rate. The common liver resection strategies are as follows: right or left hepatectomy (resection of hepatic segments 5, 6, 7, 8 or 2, 3, 4 ± 1), right or left hepatic trisectionectomy, also called extended right or left hepatectomy (resection of hepatic segments 4, 5, 6, 7, 8 or 2, 3, 4, 5, 8 ± 1), and central hepatectomy. Bisectionectomy or more was defined as a major hepatectomy; sectionectomy or less was defined as a minor hepatectomy[38]. Currently, for those patients with Bismuth type Ⅰ and Ⅱ, the right hepatectomy with caudate lobectomy was recommended, which has been demonstrated to decrease the rate of recurrence[29]. However, for those patients with Bismuth types Ⅲ and Ⅳ lesions, the approaches varied in different institutions. Recently, Cheng *et al*[49] reported 171 patients with PHC of Bismuth types Ⅲ and Ⅳ lesions. For Bismuth Type Ⅲ lesions, right, left or central hepatectomy with caudate lobectomy was performed. For Bismuth Ⅳ lesions, the right or left hepatectomy or extended right or left hepatectomy with caudate lobectomy was conducted to increase the negative margin rates. The choice of surgical side may depend on the predominance of the tumor; however, the right trisectionectomy is indicated for centrally located tumors because of the length of each hepatic duct, the location of the hilar common bile duct in the hepatoduodenal ligament, the ease of complete caudate lobectomy and portal vein reconstruction, and the frequent involvement of the right hepatic artery[7,28]. The left hepatectomy is considered to be a more complicated procedure than the right hepatectomy and requires greater skill, especially in cases involving portal vein resection and reconstruction. Moreover, preserving the right hepatic artery and the right portal vein could be an oncological problem with left or extended left resection, which could increase the tumor cell dissemination. Therefore, the rate of left hepatectomies is approximately 25%-30% of all resections[60]. In the study by Shimizu *et al*[43], the R0 resection was achieved in all 7 patients who underwent right trisectionectomy, but in only 8 (61.5%) of 13 patients who underwent left trisectionectomy. This finding suggests that a more extended resection from the right side, but not from the left side, may provide greater potential for curability. However, several authors believed that the left extended hepatectomy could achieve the same result. Nagino *et al*[51] analyzed the patients with PHC who underwent surgery and compared the surgical strategies in different periods (Table 4). From their experience, the incidence of left hepatic trisectionectomies gradually increased while the incidence of central hepatectomies decreased. Totally, the left or extended left hepatectomy represented nearly 55% of all of the resections performed on patients with PHC.

Nimura *et al*[61] introduced the concept of routine caudate lobectomy (CL). Bilateral biliary branches of the caudate lobe are confluent with the right hepatic duct, the left hepatic duct, the confluence of these and the right posterior hepatic duct. Therefore, the caudate lobe is usually involved in PHC in 40% to 98% of patients, which indicates a need for CL[61-63]. Moreover, routine CL combined with resection had high curative resectablity rates and increased the likelihood of long-term survival for patients with advanced stage PHC[49]. Similarly, Kow *et al*[64] showed that the patients with CL had a significantly better overall survival rate of 64.0 mo compared to the survival rate of 34.6 mo in type Ⅲ PHC patients in the group without CL. Although mechanisms for CL have not been established, the outcome remains optimistic while undertaking CL in PHC.

A major hepatectomy combined with pancreatoduodenectomy, for example, hepatopancreatoduodenectomy (HPD), was routinely used in the PHC surgery in several institutions. This procedure occupied 12.9% of the total surgery cases, and was indicated in the following cases: (1) diffusely infiltrating tumors of the entire extrahepatic bile duct; and (2) downward superficial spreading, or bulky nodal metastases of the pancreatoduodenal region (Table 4)[65]. Therefore, HPB provides an important method for treating spreading unresectable cholangiocarcinoma; thus, it is now the fourth standard procedure following hepatectomy, bile duct resection, and pancreatoduodenectomy[66].

In several high-volume samples, PHC was frequently reported to metastasize via the lymphatics in 24% to 75% of the patients[42,51]. Moreover, many authors had demonstrated that lymph node metastasis had a negative impact on survival in PHC[3,28,29,31,33,42,51]. Thereafter, lymphadenectomy played a crucial role in the outcome of patients with PHC. However, the 5-year survival rate is related to the location of the metastasis of the lymph node. Therefore, lymph node metastasis that is confined to the hepatic pedicle or the hepatoduodenal ligament is not a reason for abandoning resection. The tumor positive lymph nodes along the common hepatic artery or celiac axis are usually considered a contraindication for resection[7]. Kitagawa *et al*[67] showed that, in 110 patients after resection of PHC, there was a 5-year survival rate of 31%, if the lymph nodes were negative. However, in patients suffering from a local or a para-aortic lymph node infiltration, the 5-year survival rates were 15% and 12%, respectively. Interestingly, in the same report, 12% of the patients with positive para-aortic lymph nodes who lived more than 5 years were found to have macroscopically negative nodes in surgery[67]. Although the routine lymph node dissection beyond the hepatoduodenal ligament is not generally recommended, several authors still believe that lymph node dissection is beneficial.

Due to the intimate relationship between the bile duct and vessels, PHC could usually infiltrate the portal vein and hepatic artery. The indication for portal vein

Table 4 Surgery performed according to the time period[51] *n* (%)

	Total	Time period				*P*
		Earlier period		Later period		
		1997-1990	1991-2000	2001-2005	2006-2010	
Number of patients resected	574	72	116	168	218	
Resectability	574/754 (76.1)	72/93 (77.4)	116/148 (78.4)	168/216 (77.8)	218/297 (73.4)	0.406
Type of hepatectomy[1]						< 0.001
S1,4,5,6,7,8	43 (7.5)	5 (6.9)	11 (9.5)	4 (2.4)	23 (10.6)	
S1,5,6,7,8	177 (30.8)	17 (23.6)	40 (34.5)	53 (31.5)	67 (30.7)	
S1,2,3,4,5,8,	110 (19.2)	4 (5.6)	12 (10.3)	29 (17.3)	65 (29.8)	
S1,2,3,4	187 (32.6)	27 (37.5)	35 (30.2)	68 (40.5)	57 (26.1)	
S1,4,5,8/S1,5,8/S1,4/S1	38 (6.6)	13 (18.1)	10 (8.6)	11 (6.5)	4 (1.8)	
Without hepatectomy	19 (3.3)	6 (8.3)	8 (6.9)	3 (1.8)	2 (0.9)	
Combined resection						
Pancreatoduodenectomy	74 (12.9)	9 (12.5)	13 (11.2)	20 (11.9)	32 (14.7)	0.553
Portal vein resection	206 (35.9)	23 (31.9)	36 (31.0)	58 (34.5)	89 (40.8)	0.116
Wedge resection	36	15	6	10	5	
Segmental resection	170	8	30	48	84	
Hepatic artery resection	76 (13.2)	0	5 (4.3)	25 (14.9)	46 (21.1)	< 0.001
Operative time, min[2]	668 ± 134	664 ± 162	787 ± 170	675 ± 145	605 ± 134	< 0.001
Blood loss, mL[2]	2491 ± 2156	4414 ± 2791	3773 ± 3024	1898 ± 1268	1768 ± 1130	< 0.001
Homologous blood transfusion	271 (47.2)	68 (94.4)	93 (80.2)	46 (27.4)	64 (29.4)	< 0.001

Homologous blood includes packed red blood cell and fresh-frozen plasma. Note that P indicates the statistical difference between the earlier period (1977-2000) and the later period (2001-2010). [1]Expressed as Couinaud's hepatic segments resected; [2]Excluding 19 patients who did not undergo hepatectomy.

resection (PVR) and reconstruction for PHC is controversial. Previously, tumors involving the portal vein were considered unresectable. However, more recently, several surgeons have advocated this approach and its clinical benefit has been validated in many studies[22,28,29,31,33,42,50]. de Jong *et al*[50] reported the results of the analysis of an international, multicenter database from seven major hepatobiliary centers. They found that the PVR for PHC was associated with a greater risk for 30-d and 90-d perioperative mortality. Nevertheless, they thought that PVR should be undertaken, when necessary, to extirpate all of the disease because of its association with long-term survival in several patients with PHC[50]. Similarly, Nanigo recommended that PVR should be performed only when the vessel adhered to and could not be freed from the tumor during the skeletonization resection of the hepatoduodenal ligament and that PVR should not be performed as a routine procedure because it lacked scientific validation[68]. Because of the short distance between the tumor and the portal vein, Neuhaus *et al*[22] proposed a "no-touch" concept in 1999 and recommended routine PVR to achieve a wider distal radicality. Additionally, Neuhaus *et al*[69] proposed a survey to compare the effect of the "no-touch" resection with the traditional curative resection. The 5-year survival rate was significantly higher in the "no-touch" group at 58% compared to 29% in the traditional curative resection group ($P = 0.021$). However, this new technique has not been accepted by many institutions because it lacks scientific validation and more random studies are warranted for additional investigation.

In earlier reports, few institutions proposed the surgical strategy of hepatic resection combined with hepatic artery resection in patients with advanced PHC. In small samples, the outcome and survival rates were disappointing. Therefore, many authors did not recommend this surgical strategy[43,62,70]. Shimizu *et al*[43] showed that all of the nine patients undergoing left-sided hepatectomy combined with hepatic artery resection lived less than 3 years, and they considered that the hepatic artery resection was a primary prognostic factor (RR = 3.063; 95%CI: 1.289-7.282). However, in 2010, the Nagoya Institute reported their experiences with major hepatectomies with simultaneous resections and reconstructions of the portal vein and hepatic artery; the investigators showed that the challenging surgery could be performed with an acceptable mortality rate of 2% and offered a better likelihood of long-term survival with a 5-year survival rate of 30%[71]. Currently, the number of patients undergoing hepatic artery resection has been increasing (Table 4). In the institute's published data of 107 patients, the majority of patients (95%) underwent left-sided hepatectomies, of which 59% were left trisectionectomies and 36% were left hepatectomies. The overall mortality rate was 2.8% and the 5-year survival rate was 34.1%. The resected hepatic arteries were reconstructed primarily by end-to-end anastomosis, with an arterioportal shunt or an interposition graft using the radial artery or great saphenous vein[68]. For those patients who are unable to undergo hepatic artery reconstruction after resection, portal vein arterialization (PVA) could be a new approach. Using this method, adequate oxygen delivery to hepatocytes and biliary ducts can be assured. Moreover, several animal experiments showed that PVA could promote hepatic cell proliferation and enhance liver regeneration after extended hepatic resection[72]. The clini-

cal significance of hepatic artery resection is debatable, yet also promising and encouraging.

Morbidity and mortality

In Table 3, we summarize the morbidity and mortality which show significant variations, ranging from 14% to 76% and from 0% to 18%, respectively. Sano *et al*[73] defined the complications as major when they resulted in organ failure or required another surgery or interventional radiology, such as liver failure, lung failure and renal failure[51,73]. Complications that were classified as minor include pleural effusion necessitating thoracocentesis, wound infection, intra-abdominal infection with positive culture of the drainage fluid, delayed gastric emptying, anastomotic leakage, clinically silent pancreatic fistula with amylase-rich serous fluid or contaminated fluid with positive culture, and bile leakage from the raw surface of the liver healing spontaneously or responding to conservative management[73]. The most common complications observed in most institutions were infective complications, especially during earlier years of the use of these procedures, representing 50% or more of the observed complications[3,15,36]. Nagiono *et al*[51] compared the complications between the earlier years and the more recent years, and they demonstrated that the incidence of grade C liver failure, which is clinically serious, decreased markedly from 18.2% from 1977 to 1990 to 3.2% from 2006 to 2010. Wound sepsis was the second most common complication, followed by intra-abdominal abscess and bile leakage[51].

The operative mortality included all in-hospital deaths as defined by Sano. All postoperative complications that affected the outcome or lengthened the hospital stay were considered. Death may be associated with acute liver failure after extended right hepatectomy and combined portal vein resection, and sepsis with multiorgan failure[45]. Overall, these extended liver as well as vascular resections were found to be significant predictors of increased mortality[23]. In addition to liver function, operative time and blood loss may be associated with mortality[51]. Several reports have demonstrated that preoperative portal vein embolization may decrease mortality even with extended hepatectomy[73].

Outcomes and recurrence

The average 5-year survival rates after resection for PHC range from 11% to 42% (Table 3). Factors associated with favorable outcome include the following: R0 resection, no lymph node metastasis, absence of perineural and perivascular invasion, and well-differentiated histological grade. Complete resection with negative histologic margins is the only modifiable factor and, for that reason, the primary aim of surgical therapy. Recently, several reports demonstrated that patients undergoing R1 resection (microscopically positive margin) had a longer overall survival rate than patients with unresectable PHC[36,74]. Moreover, patients undergoing R0 resections with a margin less than 5 mm had the same survival rate as those patients undergoing R1 resections[29]. The surgeons were encouraged to perform more aggressive surgery to achieve a better outcome.

Few studies have analyzed recurrence patterns and time to recurrence in patients with PHC. In several reports, tumor recurrence rates can be as high as 50% to 76%, and the median time to recurrence rates has been reported to be 12 to 43 mo[36,47,75,76]. The most common site of recurrence is a local site, followed by the liver, lymph node, peritoneum and other organs. Only histologic grade was associated with recurrence-free survival[47]. Generally, the patients with recurrent disease are not candidates for curative therapy and can only receive adjuvant therapy to improve long-term outcome.

ORTHOTOPIC LIVER TRANSPLANTATION

Theoretically, orthotopic liver transplantation (OLT) offers the advantage of the resection of all of the structures that may be affected by tumor, for example, the portal vein, bilateral hepatic ducts and atrophic liver lobes. Compared to surgical resection, OLT has several advantages: (1) patients with Bismuth Ⅳ type lesions and peripheral vascular lesions cannot undergo resection; (2) patients with PHC arising from primary sclerosing cholangitis (PSC) will tolerate resection poorly because of the underlying liver impairment; (3) dissection in the hepatic hilum has the potential for causing spillage, which is an adverse prognostic factor; and (4) a clear circumferential margin is usually not achievable, which might increase the recurrence rates of PHC[77]. However, in the early years of the application of this procedure, the results were disappointing. The Cincinnati Transplant Tumor Registry collected global data between 1968 and 1997. The 1-, 2-, and 5-year survival rates were 72%, 48%, and 23%, respectively. Eighty four percent of the patients had a recurrence within 2 years of transplantation[78]. This undesirable result may have been associated with the unselected patients who had distant metastasis. Despite this finding, PHC was considered to be a relative contraindication to OLT due to the lack of organs. Interestingly, several investigations found that those patients with negative margins in transplantation and the absence of regional lymph node metastases had a better survival rate. Moreover, 22% of the patients receiving radiotherapy and chemotherapy alone had a 5-year survival, which inspired several surgeons to explore a new OLT approach for PHC.

From 1987 to 2000, Miyazaki *et al*[70] collected 17 patients who were treated with systemic chemotherapy and intraluminal bile duct irradiation as they awaited liver transplantation. Eleven patients underwent liver transplantation, and until 2000, five patients were alive without evidence of tumor recurrence with a median follow-up of 7.5 years (range, 2.8-14.5 years). In 1994, the Mayo Clinic developed a protocol employing preoperative chemoradiation therapy followed by liver transplantation, which showed encouraging results. Currently,

Table 5 Criteria for neoadjuvant therapy and liver transplantation[82]

Diagnosis of cholangiocarcinoma
Transcatheter biopsy or brush cytology
CA-19.9 > 100 mg/mL and/or a mass on cross-sectional imaging with a malignant appearing stricture on cholangiography
Biliary ploidy by FISH with a malignant appearing stricture on cholangiography
Unresectable tumor above cystic duct
Pancreatoduodenectomy for microscopic involvement of the common bile duct
Resectable cholangiocarcinoma arising in PSC
Radial tumor diameter ≤ 3 cm
Absence of intra- and extrahepatic metastases
Candidate for liver transplantation

PSC: Primary sclerosing cholangitis.

according to the Mayo Clinic protocol, patients receive EBRT (a target dose of 4500 cGy) with protracted venous infusion of 5-FU (225 mg/m^2 per day). Following this treatment, transcatheter iridium-192 brachytherapy (a target dose of 2000 cGy) is administered. Subsequently, the patients receive oral capecitabine (1000 mg/m^2 per day in two divided doses) until the time of OLT. Importantly, a staging laparotomy is performed on all of the patients before OLT to rule out metastatic disease. Only the patients with negative staging operations are eligible for transplantation[79]. Although there is a high dropout rate as patients await liver transplantation, the 5-year survival rate could achieve approximately 65% to 70%. However, the majority of patients undertaking OLT were diagnosed with PSC, and only 58% patients had histologically proven cancer which limited the use of OLT[80].

In 1996, Pichlmayr *et al*[81] proposed the indications for OLT in patients with PHC as follows: (1) unresectablity in presumed UICC stage Ⅱ confirmed by laparotomy; (2) status postresection with the intention for R0 with R or R2 positive resection margins due to advanced central tumor infiltration; and (3) local intrahepatic recurrence. After additional exploration and analysis of PHC, the Mayo Clinic proposed their criteria for neoadjuvant therapy and liver transplantation[82] (Table 5). These types of patients would be excluded if they had the following: (1) intrahepatic cholangiocarcinoma; (2) uncontrolled infection; (3) prior radiation or chemotherapy; (4) prior biliary resection or attempted resection; (5) intrahepatic metastases; (6) evidence of extrahepatic disease; (7) history of other malignancy within 5 years; and (8) transperitoneal biopsy[82]. Although the Mayo Clinic protocol has been accepted in the majority of institutions, the role of OLT requires additional substantial evidence and data confirmation from multiple institutions.

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Helicobacter pylori as a risk factor for central serous chorioretinopathy: Literature review

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Abstract

Helicobacter pylori (*H. pylori*), a Gram-negative bacterium, is one of the most frequent causes of gastrointestinal infections worldwide. It has been associated as a pathogen for the human body with many systemic diseases, including different eye diseases. We will focus on a specific eye disease called idiopathic central serous chorioretinopathy (ICSCR). This disease is characterized by a serous detachment of the neurosensory retina in the macular region, which affects the vision to different degrees. Currently, the pathophysiology of ICSCR is not clear and there is no effective treatment. However, several potential risk factors have been elucidated. One of the factors that has more frequently been associated with ICSCR is stress. As *H. pylori* was identified as a possible etiological factor for occlusive arterial diseases in young people who were particularly stressed, it was thought that *H. pylori* might also be present in ICSCR. Therefore, some physicians started to test its presence in patents with ICSCR. If *H. pylori* happened to be associated with ICSCR, the treatment of gastrointestinal infection could also improve visual symptoms and help to remediate this eye disease. Although *H. pylori* is highly prevalent in the general population, a true correlation seems to exist. We present a review on the relationship between ICSCR and *H. pylori*.

Key words: *Helicobacter pylori*; Idiopathic central serous chorioretinopathy; Retina; Eye disease; Occlusive arterial disease

Core tip: *Helicobacter pylori* (*H. pylori*) has been associated with many systemic diseases. We focus on a specific eye disease called idiopathic central serous chorioretinopathy (ICSCR), which is characterized by a serous detachment of the neurosensory retina in the macular region and affects vision to different degrees. One factor frequently associated with ICSCR is stress. As *H. pylori* was identified as a possible etiological factor for occlusive arterial diseases in young people who were particularly stressed, it was thought that *H. pylori* might also be present in ICSCR. We present a review on the relationship between ICSCR and *H. pylori*.

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INTRODUCTION

Helicobacter pylori (*H. pylori*), a Gram-negative bacterium, is one of the most frequent causes of gastrointestinal infections worldwide. It has been associated as a pathogen for the human body with many systemic diseases, including vascular (atherosclerosis and cardiovascular diseases, Raynaud's syndrome, primary headache), autoimmune (Sjögren syndrome, autoimmune thyroiditis, idiopathic arrythmias, Parkinson's disease, nonarterial anterior

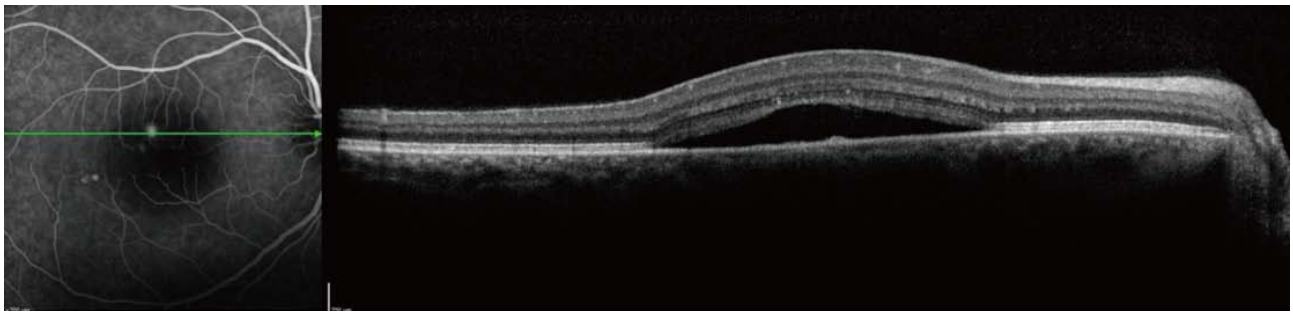

Figure 1 Optical coherence tomography image showing separation of the sensory retina from the retinal pigment epithelium.

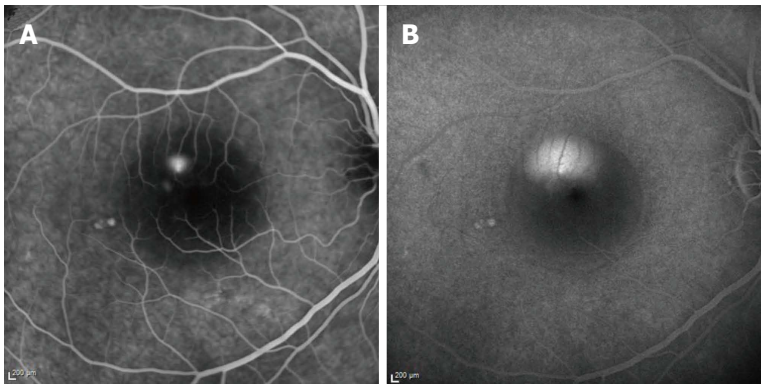

Figure 2 Fluorescein angiography at 2 (A) and 20 (B) min. A: The early phase shows a hyperfluorescent spot due to leakage of dye through the RPE; B: During the late venous phase, fluorescein passes into the subretinal space and spreads until the entire area is filled with dye.

optic ischemic neuropathy) and skin diseases (urticaria, rosacea), iron deficiency anemia, growth retardation, late menarche, extra-gastric MALT lymphoma, duodenal ulcer, gastric cancer, gastro-oesophageal reflux disease, diabetes mellitus, hepatic encephalopathy, sudden infant death syndrome, and anorexia of aging[1-4].

H. pylori has also been associated with eye diseases such as Sjögren syndrome, blepharitis, glaucoma, uveitis and idiopathic central serous chorioretinopathy (ICSCR)[5-9].

Our review focuses on the relation between ICSCR and *H. pylori*. ICSCR was first described by von Graefe in 1886[10]. ICSCR affects middle-aged adults (between 25-45 years old), predominantly men, and is characterized by a serous detachment of the neurosensory retina in the macular region. It is usually unilateral (90% of the patients). Patients may develop metamorphopsia, central positive scotoma, micropsia, and impaired color vision. Additional retinal findings include retinal pigment epithelium (RPE) detachment, RPE atrophic tracks, capillary telangiectasia, retinal or choroidal neovascularisation, and intraretinal deposits[11-13], which may be visualized with fluorescein and indocyanine green angiography, and optical coherence tomography (OCT). (Figures 1 and 2).

Most of the cases spontaneously resolve with recovery of good visual function. However, recurrences have been observed in 50% or more of the cases[14]. A small percentage of subjects experience chronic decompensation of the RPE and develop severe vision loss.

The pathophysiology of ICSCR is poorly understood. It is thought that damage to the RPE active fluid transport mechanisms that usually dehydrate the subretinal space may play a role[14]. Cigarette smoking, systemic hypertension, pregnancy, allergic respiratory disease, antibiotic or alcohol ingestion[15], sildenafil citrate[16] or systemic corticosteroids[17], sympathomimetic agents[18], antiphospholipid antibodies[19], retinitis pigmentosa[20], psoriasis[21], and endogenous mineralcorticoid dysfunction[17] have been cited as potential risk factors for this disease. ICSCR has also been reported in patients with a benign tumor of the adrenal gland[22], cryoglobulinemia[23], systemic lupus erythematosus[24], or after bone marrow transplantation[25]; and has been strongly associated with individuals with type A personality[26].

Currently, there is no effective treatment for ICSCR. Photodynamic therapy with verteporfin has been used in the last few years. Although it decreases serous detachment and improves visual acuity, it results in scotomas in some patients. A new treatment has recently been proposed based on oral eplerenone. Experimental data has shown that central chorioretinopathy could result from an overactivity of the mineralcorticoid receptor pathway in choroid vessels. Eplerenone is a mineralcorticoid receptor antagonist and has therefore been considered as a potential treatment for ICSCR. Randomized controlled trials are needed to confirm if this therapy could help in the treatment of ICSCR[17].

DISCUSSION

HP was first associated with ICSCR in 2000. A French team (Mauget-Faÿse *et al*[7]) presented their first results on a poster at the Association for Research in Vision and Ophthalmology (ARVO) congress. Knowing that

H. pylori was identified as a possible etiological factor for occlusive arterial diseases in young people who were particularly stressed[27], the authors of this study decided to test the presence of HP infection in ICSCR patients. As occlusive arterial disease shared some characteristics with ICSCR (*i.e.*, associated with type A personality and ischemia), it was believed that HP infection might be a common factor.

This prospective pilot study of 16 patients affected by active ICSCR or by its variant, diffuse retinal epitheliopathy, found that the prevalence of *H. pylori* infection; determined by one of more of the following methods: histology of gastric biopsy specimens, C-urea breath test, or serology test (Boehringer Mannhein test); was significantly higher in subjects with ICSCR[7]. A complementary study including more patients and confirming the results was published in 2004[28]. A few months earlier, a case report of a 43-year-old man suggested that ICSCR recurrences were associated with the presence of *H. pylori*. Resolution of ICSCR was correlated with the eradication of the bacterium using the conventional triple-therapy regimen (amoxicillin, clarithromycin, omeprazole)[29].

Some further studies confirmed this relationship. A Spanish team observed that 68.75% of ICSCR patients were infected with *H. pylori*, compared with 30% of the control population[30]. Recently, Casella *et al*[18] suggested that chronic ICSCR patients could be infected with *H. pylori* and that the treatment of the infection could have a positive impact on the outcome of chronic ICSCR regarding the improvement of final best-corrected visual acuity and resolution of the serous detachment. Lastly, Dang *et al*[17] reported that, although *H. pylori* eradication does not increase visual acuity and does not diminish subretinal fluid, it could benefit central retinal sensitivity in ICSCR patients. A statistical difference was observed in central retinal sensitivity at 3 mo after HP eradication therapy. Macular sensitivity was measured using microperimeter-1 (Nidek, Vigonza, Italy) after pupil dilatation and not with contrast sensitivity charts. Thirty-three stimulus points located in the area of the central 15° diameter around the macula were examined. The average sensitivity of the 33 points was defined as the central retinal sensitivity[17].

It is difficult to determine the potential role of *H. pylori* in the pathogenesis of ICSCR. Giusti elucidated several hypotheses regarding pathogenesis[31]. A possible explanation might be the link between *H. pylori* infection and atherosclerosis. A cross reactivity of anti-Cag A antibodies, whose presence is more frequently associated in atherosclerosis, and the presence of immunoglobulin-G (Ig-G) antibody have been considered as risk factors for endothelial dysfunction[32]. Another mechanism is the role of heat shock proteins expressed by several pathogens, *e.g.*, *H. pylori*. It has been hypothesized that an immune response against antigens located on pathogenic organisms would cross-react with homologous host proteins, *e.g.*, with the endothelial vascular wall[33].

Further implications of *H. pylori* infections have lately been proposed: increase of lipids and fibrinogen levels[32], upregulation of endothelial adhesion molecules and increase of polymorphonuclear leucocyte adhesion[34], and increase of platelet activation and aggregation[35].

CONCLUSION

Several studies indicate that many ICSCR patients could be infected with *H. pylori* and that the treatment of the infection could have a positive impact on the outcome of the disease. Due to the high prevalence of *H. pylori* infection in the general population, it is difficult to establish a true correlation. Prospective and masked clinical trials are necessary to confirm the relationship between ICSCR and *H. pylori*, as well as the benefits to ICSCR patients from receiving *H. pylori* treatment.

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MINIREVIEWS

Risk of cardiovascular disease in inflammatory bowel disease

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Abstract

Abundant scientific evidence supporting an association between inflammatory bowel disease (IBD) and venous thromboembolic events, caused by an IBD related hypercoagulability, is acknowledged and thromboprophylactic treatment strategies are now implemented in the management of IBD patients. In contrary, the risk of arterial thromboembolic disease, as ischemic heart disease, cerebrovascular events, and mesenteric ischemia in patients with IBD remains uncertain and the magnitude of a potentially increased risk is continuously debated, with ambiguous risk estimates among studies. The evident role of inflammation in the pathogenesis of atherosclerosis forms the basis of a biological plausible link; the chronic systemic inflammation in IBD patients increases the risk of atherosclerosis and thereby the risk of thrombotic events. Further, studies have shown that the burden of traditional risk factors for atherosclerosis, such as obesity, diabetes mellitus, and dyslipidemia is lower in IBD populations, thus further strengthen the role of non-traditional risk factors, as chronic inflammation in the linking of the two disease entities. Likewise, mortality from cardiovascular disease in IBD remains questioned. The aim of the current review is to give an up-date on the existing evidence of the possible association between IBD and cardiovascular disease and to discuss traditional and non-traditional risk factors.

Key words: Inflammatory bowel disease; cardiovascular disease; Risk; Ulcerative colitis; Crohn's disease

Core tip: The increased risk of venous thromboembolic events in inflammatory bowel disease (IBD) patients is well-established and prophylactic strategies are implemented in current guidelines. The risk of arterial thromboembolic complications in IBD remains uncertain. Together, the systemic inflammation in patients with IBD and the inflammation-driven development of atherosclerosis form the basis of a potential association between the two disease entities. The present review will provide a summary of the existing literature on the association between IBD and thromboembolic diseases and discuss potential risk and preventive factors.

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INTRODUCTION

Inflammatory bowel disease (IBD), comprising ulcerative colitis (UC) and Crohn's disease (CD) are systemic, chronic inflammatory conditions that predominately affect the gastrointestinal tract but are also characterized by numerous extraintestinal manifestations, assumedly caused by concomitant systemic inflammation. It is well-established that the risk of venous thromboembolic event is increased in IBD patients[1], primarily during flares[2], potentially due to an inflammation induced state

of hypercoagulability. However, the true magnitude of this risk and the associated mortality rate remains debated.

In the last decade, it has become increasingly evident that chronic systemic inflammation plays a pivotal role in the pathogenesis of atherosclerosis[3]. Further, the observation of increased thickness of the carotid intimal-media (a measure of atherosclerotic burden), endothelia dysfunction, and atherogenic alterations in the lipid profile of patients with IBD has further fuelled the hypothesis of a potential increased risk of atherosclerosis-driven vascular diseases in IBD[4-6]. Likewise, an increased risk of cardiovascular diseases (CVD) in other inflammatory conditions as rheumatoid arthritis[7], psoriasis[8] and systemic lupus erythematous[9] is now established, independent of traditional cardiovascular risk factors. Currently, reported results on risk of CVD in IBD have been ambiguous with studies revealing an increased risk of both ischemic heart disease (IHD) and cerebrovascular accidents (CVA) while others have shown no association[10-13]. Additionally, a few studies have suggested that IBD patients have a lower burden of some of the traditional risk factors for CVD, such as hypertension, diabetes mellitus, dyslipidemia, and obesity, and that non-traditional risk factors could play an important role for IBD patients[13,14]. Overall, this has led to an ongoing debate of whether the risk of arterial thrombotic disease is increased in IBD patients, what the underlying mechanisms are, and whether a strategy for disease specific risk assessment should be implemented in the management of IBD patients.

The aim of the current review is to give an update on the existing evidence on risk of atherosclerosis-related vascular disease, including ischemic heart disease, cerebrovascular accidents, mesenteric thrombosis, and venous thromboembolic events and associated risk factors and mortality rates in patients with IBD and further to evaluate on future prospects and preventive factors.

VENOUS THROMBOEMBOLIC EVENTS

The association between venous thromboembolic events (VTEs), comprising deep venous thrombosis (DVT) and pulmonary embolism (PE), and IBD was indicated as early as in 1936 by Bargen *et al*[15]. In 1986, fifty years after the suggested association, Talbot *et al*[16] was the first to report valid results on the incidence of VTE's in 7199 IBD patients from the Mayo Clinic, US and revealed a potentially increased risk.

VTEs are a serious concern with a significant morbidity and mortality. The risk of VTEs is associated with the hypercoagulability related to IBD. The specific clotting mechanism have been attributed to a range of factors including thrombocytosis[17], increased levels of clotting factors V/Ⅷ/fibrinogen[18], acquired antithrombin Ⅲ deficiency[19,20] and decreased levels of protein C and S[21-23]. The exact mechanism, the interplay between the variable factors and whether the hypercoagulability is a secondary phenomenon to IBD or represents an underlying pathological mechanism for IBD remain uncertain.

In 2001, the first large population-based study on risk of VTE's in IBD was reported from the Canadian Manitoba database. In a cohort of 5,529 IBD patients matched 1:10 with healthy controls from the general population, the risk of DVT and PE was significantly increased in IBD patients compared to controls (incidence rate ratio, IRR = 3.54, 95%CI: 2.9-4.3; and IRR = 3.3, 95%CI: 2.5-4.3 for DVT and PE respectively). IBD patients < 40 years of age were at particular high risk of VTEs with a six-fold increased risk (IRR = 6.02; 95%CI: 3.92-9.12). No sex or IBD subtype differences were observed[24]. This study led to the introduction of thromboprophylaxis as the standard care for IBD patients with active inflammation admitted to hospital. A later population-based study from the United Kingdom by Grainge *et al*[2] sought to elucidate the risk of VTE's during different stages of disease activity as they hypothesized that the more severe inflammation the greater risk of VTEs. In 13756 IBD patients, matched with 71627 non-IBD controls, the risk of developing VTE's was similar to the results from Canada with a hazard ratio (HR) of 3.4 (95%CI: 2.7-4.3). Further the study found that the risk of VTEs during a flare (defined as the period 120 d after a new corticosteroid prescription) was much more prominent with a HR of 8.4 (95%CI: 5.5-12.8). The highest relative risk of VTEs was found for IBD patients non-hospitalized during a flare with an almost 16-fold increased risk (HR = 15.8; 95%CI: 9.8-25.5). A recent meta-analysis identified 10 studies assessing the risk of VTEs in 72205 IBD patients and 891840 controls and found that the overall risk of VTEs in IBD was increased by 96% compared to the general population (RR = 1.96; 95%CI: 1.67-2.30)[25]. No difference in risk was found between UC and CD. The meta-analysis further confirmed that the risk of VTEs was greater in studies including IBD patients in general (RR = 2.48; 95%CI: 2.04-3.00) compared to studies evaluating on hospitalized IBD patients (RR 1.47; 95%CI: 1.17-1.86). This observation is potentially due to an effect of thromboprophylactic treatment strategies for hospitalized IBD patients.

Only few studies have evaluated on mortality rates in VTE complicated IBD patients. From the Mayo Clinic, Solem *et al*[26] reported a 22% mortality rate after a median follow-up of 1.8 years among 98 IBD patients diagnosed with VTE however, no comparison was made with post-VTE mortality rates in the general population. A large nation-wide population-based study from the United States by Nguyen and Sam[27], including more than a hundred thousand IBD patients, revealed that the in-hospital mortality was significantly higher for IBD patients with VTE compared with non-VTE IBD patients and this was valid for both CD (17.0 *vs* 4.2 per 1000 hospitalizations, $P < 0.0001$) and UC (37.4 *vs* 9.9 per 1000 hospitalizations, $P < 0.0001$). The excess mortality associated with VTE was 2.1 fold higher for

IBD patients than non-IBD individuals with VTEs ($P < 0.0001$) thereby indicating that VTEs have a more severe prognosis in IBD patients than in non-IBD individuals.

To summarize, it appears evident that IBD is a moderate independent risk factor for the development of VTEs and that the risk is highest among IBD patients with a flare in disease, not admitted to hospital. Further, there is a significant mortality associated with VTEs in IBD patients that is even greater than in non-IBD patient with VTEs. This calls for the importance of preventative and treatment strategies of VTEs in the IBD population, especially in the light of results from a recent survey involving 591 United States physicians; only 35% would give pharmacologic VTE prophylaxis to a hospitalized patient with severe UC[28].

ARTERIAL THROMBOEMBOLISM

In contrast to the well-established association between IBD and VTE, the risk of arterial thromboembolic events (ATE) in IBD is less elucidated in the literature. In the following, for simplicity, ATE will comprehend ischemic heart disease (IHD), cerebrovascular disease (CVD) and mesenteric ischemia.

Several circumstances could suggest that IBD patients are at increased risk of ATE. First of all IBD patients, particular CD patients are more likely to be current or past smokers. Further, some IBD-related drugs, *e.g.*, corticosteroids which increases the blood pressure and change the glucose homeostasis, and in contrary, the avoidance of aspirin-containing medications (due to potential fear of exacerbating IBD) could potentially increase the risk of ATE in IBD. Additionally, the presence of a chronic systemic inflammation in IBD, a well-known independent risk factor for atherosclerosis, assumedly augments the risk.

ISCHEMIC HEART DISEASE

Ischemic heart disease is caused by atherosclerotic plaque formation in coronary arteries and it is the most common type of heart disease and the leading cause of death in the world. Several inflammatory mediators as high C-reactive protein, and further up-stream inflammation markers such as tumor necrosis factor-α, interleukin-6 and 18 and the CD40 ligand are involved in the pathogenesis of both chronic inflammatory conditions including IBD and atherosclerosis[17,29]. Further, studies have revealed that IBD patients, compared to non-IBD individuals, have an increased carotid intima-media thickness, a surrogate marker for IHD and have a higher risk of early onset of atherosclerosis[6,30]. Thus, it appears biologically plausible that IBD patients carry an augmented risk of IHD compared to the general population.

In 2008, the first large study on risk of IHD in IBD patients, a population-based study from the Manitoba Database, Canada conducted by Bernstein *et al*[31], reported a 26% increased risk (IRR = 1.26; 95%CI: 1.11-1.44) of IHD in 8060 IBD patients compared to non-IBD individuals. No difference in risk was observed between sex and subtype of IBD.

In contrary, a retrospective matched cohort study from United States by Ha *et al*[10] including 17487 IBD patients did not reveal any overall increased risk of IHD in either CD or UC, but in sub-analyses the risk of myocardial infarction was significantly increased in IBD women aged above 40 years (HR = 1.16; $P = 0.003$).

In a matched cohort study by Yarur *et al*[13] from 2011, the risk of IHD was assessed among 356 IBD patients and 712 matched controls and the authors reported a nearly 3-fold increased risk of IHD in IBD (HR = 2.85; 95%CI: 1.82-4.46). A nationwide Danish population-based cohort study of 4570820 individuals by Rungoe *et al*[12] reported a lower, although significant increased risk of IHD (IRR = 1.59; 95%CI: 1.50-1.69) in IBD patients compared to non-IBD individuals[12]. Analyzing risk of IHD solely in the first three months and during the first year after IBD diagnosis revealed particularly high risk estimates (IRR = 4.57; 95%CI: 3.89-5.36 and IRR = 2.13; 95%CI: 1.91-2.38 respectively), hence also reflecting the potential role of ascertainment bias when assessing two chronic diseases (*i.e.* that hospitalization for one of the diseases increases the potential for discovery and recording of the other disease). However, analyses disregarding the first year after diagnosis and fully adjusted for co-morbidity related medications revealed a persistent 22% increased risk of IHD over time (IRR = 1.22; 95%CI: 1.14-1.30). A following population-based Danish study by Kristensen *et al*[11] reported risk of myocardial infarction (MI) in more than 20.000 IBD patients according to disease activity. Analyses revealed an increased risk of MI in IBD patients during flare (RR = 1.49; 95%CI: 1.16-1.93) and during persistent activity (RR = 2.05; 95%CI: 1.58-2.65), whereas the risk was not increased during periods of remission (RR = 1.01; 95%CI: 0.89-1.15). In accordance with the Danish findings, a meta-analysis on risk of IHD in IBD by Singh *et al*[32] reported a 19% increased risk of IHD in IBD patients (OR = 1.19; 95%CI: 1.08-1.31) with the risk being higher in female gender (OR = 1.26; 95%CI: 1.18-1-35). Interestingly, another meta-analysis by Fumery *et al*[25], solely including observational studies on risk of IHD in IBD did not (potentially due to lack of power) reveal a statistically increased risk, although the magnitude of risk was similar (RR = 1.23; 95%CI: 0.94-1.62). The main difference between the two meta-analyses was the inclusion of a cross-sectional study by Sridhar *et al*[33] only in the latter meta-analysis; a study that contrary to expected found an inverse association between IHD and hospitalized IBD patients with a significant protective effect of IBD on risk of IHD (OR = 0.60; 95%CI: 0.56-0.65). With results paradoxical to the hypothesis authors explained this protective association could be caused by a direct result of Berkson's fallacy[34], a form of selection bias that causes hospital cases and non-hospital controls in a case control study to be systemati-

cally different from one another, leading to a systematically higher exposure rate among hospital patients, and thereby distorting the risk estimate.

CEREBROVASCULAR DISEASE

Several case reports of ischemic stroke in remarkably young patients with CD has additionally led to the hypothesis of a potential association between IBD and CVE[35-38].

Bernstein and colleagues reported a slightly increased risk of cerebrovascular disease in patients with CD (but not UC) in a population based setting (IRR = 1.32; 95%CI: 1.05-1.66), but adjustments were insufficient, lacking several important cerebrovascular risk factors, such as smoking, obesity and hypertension[31]. A population-based case-control study from the United States evaluated on risk of ischemic stroke among 8054 CD patients matched with 161078 non-CD patients and results revealed an insignificant overall increased risk of ischemic stroke (OR = 1.10; 95%CI: 0.85-1.43)[39]. A significant almost 3-fold increased risk of ischemic stroke was estimated in younger CD patients below 50 years of age (OR = 2.93; 95%CI: 1.44-5.89). A large United States conducted population-based matched cohort study found no overall increased risk of cerebrovascular disease in IBD patients, but stratified analyses revealed a significantly increased risk of stroke among women with IBD below the age of 40 compared to non-IBD controls (HR = 2.1, $P < 0.05$)[10]. Only in a Danish setting an overall slightly increased risk of stroke in IBD patients has been estimated (RR = 1.15; 95%CI: 1.04-1.27)[11] and during flares this risk was further increased (RR = 1.53; 95%CI: 1.22-1.92).

The meta-analysis by Singh *et al*[32] reported pooled OR from five studies on cerebrovascular events in IBD and the meta-analysis revealed an adjusted 18% increased risk of CVE in IBD (OR = 1.18; 95%CI: 1.09-1.27), with a higher magnitude of risk estimates in women and patients at younger age.

INTESTINAL ISCHEMIA

The association between intestinal ischemia (including acute/chronic mesenteric ischemia and ischemic colitis) and IBD is vaguely elucidated. A population-based case-control study from the United Kingdom from 2011 studied risk factors for intestinal ischemia from the General Practice Research Database (GPRD)[40]. Of the 71 cases of intestinal ischemia derived from the database only one patient had intestinal ischemia and IBD corresponding to an insignificant 4-fold increased risk (OR = 4.19; 95%CI: 0.46-38.43). From the Nationwide Inpatient Sample (NIS), the largest inpatient database in the United States, the risk of mesenteric ischemia was assessed among nearly 150000 discharges with a diagnosis of IBD and revealed a significant association between IBD and mesenteric ischemia (adjusted OR = 3.4; 95%CI: 2.90-4.00) with a higher risk among UC patients (OR = 5.3; 95%CI: 4.24-6.74) than CD patients (2.58; 95%CI: 2.09-3.17). Young females with UC in the age group from 18 -39 years had the highest risk (OR = 15.48; 95%CI: 8.98-26.67). Likewise, a large cohort study[10] reported increased risk of mesenteric ischemia in IBD patients with a HR of 11.2 compared with controls ($P < 0.0001$) and found the risk to be highest in UC patients (HR = 12.5; $P < 0.0001$) and females aged between 18-39 years (HR = 22.3; $P < 0.0001$). Although the absolute risk may be limited, mesenteric ischemia remains a very serious condition and IBD practitioners should be aware of the importance of recognizing these events.

CARDIOVASCULAR MORTALITY

Several studies have assessed the mortality rate from CVD in IBD and reports on both increased and decreased mortality rates exist[41,42]. In a recent meta-analysis by Bewtra *et al*[43] of cause-specific standardized mortality ratios in both population-based and inception cohort studies of IBD patients, no increased mortality from cardiovascular disease in neither UC nor CD was found (SMR_{UC} = 0.90; 95%CI: 0.80-1.02 and SMR_{CD} = 1.00; 95%CI: 0.88-1.13). Similar insignificant risk estimates of cardiovascular mortality in IBD patients was reported in the meta-analysis by Fumery and colleges (pooled SMR = 1.03; 95%CI: 0.93-1.14)[25]. Nevertheless, it is important to keep in mind that although cardiovascular mortality is a hard end-point and less prone to ascertainment bias it does not capture the entire spectrum of cardiovascular disease and with improving therapeutic options the mortality rate is decreasing and observational studies on the association between IBD and cardiovascular mortality often does not reach statistical significance due to the low mortality rates. In the large-scale population-based study by Kristensen *et al*[11] with non-increased overall CV mortality among patients in remission (RR = 0.98; 95%CI: 0.89-1.09), authors were able to show increased CV mortality during flares (RR = 2.32; 95%CI: 2.01-2.68) and in patients with persistent disease activity (RR = 2.50; 95%CI: 2.14-2.92).

RISK FACTORS

The traditional risk factors for CVD are hypertension, diabetes mellitus, obesity, smoking, dyslipidemia, and physical inactivity.

A small Indian study by Sappati Biyyani *et al*[14] aimed at evaluating the presence of traditional atherosclerotic risk factors in patients with IBD and coronary artery disease (CAD) compared to a control group (only CAD) by using the Framingham risk score. The Framingham risk score is a 10-year risk of CAD score based on the following risk factors: age, hypertension, diabetes mellitus, tobacco use and dyslipidemia. Among 42 cases and 137 controls the Framingham risk score was significantly

lower in patients with both IBD and CAD compared to controls (8.1 *vs* 10.0; $P = 0.002$). Yarur *et al*[13] further assessed traditional and nontraditional risk factors in IBD related CAD and found that several traditional risk factors usually linked with patients' anthropometric status were less common in IBD. Kristensen *et al*[11] made subgroup analyses stratifying IBD patients according to presence of traditional risk factors and showed a strong association between the number of risk factors and the risk of cardiovascular events. Additionally it is interesting that this study found an association between disease activity and risk of CV events, thereby supporting the hypothesis that the chronic inflammation acts as a risk factor for CVD in IBD patients. This is in accordance with another Danish study by Rungoe and colleagues stratifying risk according to use of oral corticosteroids, used as a proxy for both current and later disease activity, and the study revealed a higher risk of IHD in IBD patients with a history of oral corticosteroids compared to never users (IRR = 1.37 *vs* 1.23 respectively; $P < 0.01$)[12].

POTENTIAL PREVENTIVE TREATMENTS

Considering chronic systemic inflammation as a potential nontraditional risk factor for CVD in IBD, it is interesting to evaluate the effect of treatments lowering the inflammatory burden on risk of CVD; despite the fact that anti-inflammatory therapy as treatment for atherosclerosis has received little attention. However, only few studies have addressed the impact of inflammation lowering drugs use in the management of IBD on risk of CVD.

In the study by Bewtra *et al*[44] sub-analyses stratifying between users and non-users of 5-aminosalicylic (5-ASA), a drug potentially possessing aspirin like properties, revealed a significant decreased risk of IHD in IBD patients receiving 5-ASA compared to never users (IRR = 1.16 *vs* 1.36 respectively; $P = 0.02$)[12]. Restricting analyses to long-term use of 5-ASA (defined as three or more redeemed prescriptions) further strengthened the finding of a preventive effect of 5-ASA on IHD (further decrease in IRR = of IHD to 1.08; 95%CI: 0.98-1.19). Interestingly, this observation of a preventive effect of 5-ASA on IHD was only present in IBD patients receiving oral corticosteroids which in this case was used as a proxy for disease severity. These results could indicate that only IBD patients with more severe disease or increased disease activity, are at increased risk of IHD and in this case the aspirin-like moiety of 5-ASA may have preventive properties.

As stated previously, the pro-inflammatory cytokine TNF-α plays an important role in the inflammatory process in both the intestine and in development of atherosclerosis. Accordingly, biological drugs impairing this cytokine, *e.g.*, infliximab and adalimumab, have been outlined not only as potential preventive treatments lowering the risk of CVD in IBD but also as a potential treatment for atherosclerotic disease as IHD in the general population. The direct and indirect effects of the TNF-α cytokine on the cardiovascular system is very complex and to some extend paradoxical. It is beyond the scope of the present review to give a detailed description of the pathological effects of TNF-α, but overall TNF-α tends to have both beneficial and harmful effects on the cardiovascular system, both in *in vitro* and *in vivo* studies; suggestively caused by a TNF-α concentration-related difference in effect and activation of different receptors[45-49]. This might also be the reason for conflicting results in studies evaluating the effect of TNF-α antagonist as a potential treatment option for atherosclerosis and IHD[48,50].

A study by Greenberg *et al*[51] evaluated on CV events associated with TNF-α antagonist treatment among more than 10000 patients with reumathoid arthritis (RA) and found that TNF-α antagonists treatment was associated with a reduced risk of cardiovascular events compared to RA patients treated with traditional disease-modifying antirheumatic drugs (HR = 0.39; 95%CI: 0.19-0.82). The risk of CVD, including both IHD and CVE, in IBD patients treated with TNF-α antagonists was elucidated in a Danish population-based study including more than 50000 IBD patients. Thirty-one TNF-α antagonist-exposed patients and 2641 unexposed patients developed IHD, yielding an adjusted RR of 0.85 (95%CI: 0.59-1.24) whereas the risk of CVE associated with TNF-α antagonists was 1.42 (95%CI: 0.82-2.45)[52]. Thus, point estimates indicate a protective effect of TNF-α antagonist on IHD but at the same time suggest TNF-α antagonists to be a risk factor for CVE, though noteworthy none of the estimates reached statistical significance. The complexity of TNF-α and the therapies targeting the cytokine demands for forthcoming intensive and thorough research in the field before any clear evaluation can be fulfilled.

A recent interest has been raised to the HMG-CoA-reductase inhibitors (statins), drugs mainly used for hyperlipidemia but comprise pleiotropic properties as pro-apoptotic, anti-angiogenic, and anti-inflammatory effects. The anti-inflammatory capacity of statins has been evaluated in IBD patients in a large retrospective study by Crockett *et al*[53] revealing a 18% reduction in initiation of oral steroids in IBD patients (HR = 0.82; 95%CI: 0.71-0.94) and an even greater reduction for UC patients (HR = 0.75; 95%CI: 0.62, 0.91). Future studies are needed to clarify the beneficial effect of statins in IBD and whether a potential synergetic effect may develop due to the potential of both lowering the risk of atherosclerosis and the inflammation in IBD.

CONCLUSION

The association between venous thromboembolic events and IBD is well-established and may cause significant morbidity and mortality. Although antithrombotic prophylactic treatment is recommended for hospitalized IBD patients, surveys have shown that these recommendations are by far not followed in practice and greater

attention to this issue is warranted.

Regarding arterial thromboembolic diseases, it seems plausible and it is further supported by recent literature, that the risk of CVD is increased in IBD patients, particularly during flares. The elevated risk is most likely due to an increased atherosclerotic burden triggered by inflammatory mediators, such as CRP, interleukin 6, and TNF-α.

Future large, prospective longitudinal studies are needed to determine the true risk of CVD in IBD and to further characterize preventive and risk factors. It is of particular interest whether tight control of the IBD-related inflammation could lower the progression and early development of atherosclerosis in these patients.

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RETROSPECTIVE STUDY

Cancer stem cells in *Helicobacter pylori* infection and aging: Implications for gastric carcinogenesis

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Abstract

AIM: To demonstrated the combined effects of aging and carcinogen treatment on cancer stem/stem-like cells (CSCs) of gastric mucosa in an animal model.

METHODS: In this study we investigated the effects of aging and *Helicobacter pylori* (*H. pylori*) inflammation as a model for inflammation induced carcinogenesis in human and rat gastric mucosa samples. In aging studies, we compared 4-mo old (young) with 22 mo (aged) old Fischer-344 rats. For human studies, gastric biopsies and resection specimens representing normal mucosa or different stages of *H. pylori* gastritis and gastric adenocarcinomas were used for determining the expression of stem cell markers CD166, ALDH1 and LGR5. In addition we performed immunofluorescent double labeling for B-catenin and Lgr5 in both rat and human gastric tissues to examine the status of Wnt signaling in these cells.

RESULTS: CSC markers ALDH1, LGR5, and CD166 were expressed in very low levels in normal human gastric mucosa or young rat gastric mucosa. In contrast, level of expression for all three markers significantly increased in *H. pylori* gastritis and gastric adenocarcinomas as well as in normal gastric mucosa in aged rats. We also observed cytoplasmic B-catenin staining in both aged rat and human *H. pylori* inflamed gastric mucosa, which were found to be colocalized with Lgr5 immunoreactive cells. The increased number of ALDH1, CD166 and LGR5 positive cells in *H. pylori* gastritis indicates that increased number of stem-like cells in gastric mucosa is an early event, and may constitute an important step in the progression to neoplasia.

CONCLUSION: Our observation of the age-related increase in cancer stem/stem-like cells in the gastric mucosa may explain the increased incidence of gastric cancer during aging. Combination of aging and *H. pylori* infection may have additive effects in progression to neoplasia.

Key words: Cancer stem cells; Aging; CD166; ALDH1; LGR5; Gastric cancer; *Helicobacter pylori*

Core tip: In this study we demonstrated an age-related increase in cancer stem/stem-like cells (CSCs) in normal appearing gastric mucosa with activated Wnt signaling. In addition, we have shown that gastric infection

by *Helicobacter pylori* (*H. pylori*) induces an increase in CSC population in the gastric mucosa. Based on our observations we believe that aging and chronic inflammation with *H. pylori* are two significant factors that overlap and presumably exacerbate each other in gastric carcinogenesis.

Levi E, Sochacki P, Khoury N, Patel BB, Majumdar APN. Cancer stem cells in *Helicobacter pylori* infection and aging: Implications for gastric carcinogenesis. *World J Gastrointest Pathophysiol* 2014; 5(3): 366-372 Available from: URL: http://www.wjgnet.com/2150-5330/full/v5/i3/366.htm DOI: http://dx.doi.org/10.4291/wjgp.v5.i3.366

INTRODUCTION

It has been well established that the incidences of cancer rise sharply with age and the majority of cancer cases are detected in patients over the age of 65 years[1]. Such a direct correlation between cancer incidence and advanced age in most cancers clearly suggests that the phenomenon of aging and cancer are intricately connected. Accumulating evidence also suggests that the increase in tumor incidence with advancing age is preceded in part by chronic disorders including inflammation[1,2]. The etiological causes of inflammation are many folds and include viruses, bacteria, environmental pollutants, and stress as well as food factors. Chronic inflammation as risk factor for most cancers is well recognized[2].

Aging and chronic inflammation are two factors associated with an increased risk for gastric cancer[1,2]. Within the gastrointestinal tract, inflammatory conditions such as gastroesophageal reflux disease, *Helicobacter pylori* infection (*H. pylori*), inflammatory bowel disease, and viral hepatitis are well known to be associated with cancers[1,2]. The possible explanations for the link between cancer and inflammation are accumulating gene mutations, inhibition of apoptosis, increased cell proliferation and pro-inflammatory cytokine release which creates a pro-carcinogenic microenvironment[1,2].

A growing body of evidence supports the contention that cancers, including the gastric cancer are diseases driven by a small set of self renewing cells, termed cancer stem cells (CSC) or cancer-initiating cells, that are distinct from the bulk of the cells in the tumor[3-9]. CSCs are widely believed to arise from the normal stem cells or progenitor cells upon mutations[7].

The putative progenitor/stem cell in the stomach is thought to reside in the isthmic region of the fundic epithelium[6,7,10]. In mice, granule free cells in the isthmus have been shown to act as stem cells[11].

The gastric progenitor/stem cells accomodate to acute and chronic injury to the gastric epithelium and replenish the destroyed epithelium during the lifetime of the organism of interest, which creates the risk of accumulating mutations and giving rise to gastric cancers[7,8,12]. *H. pylori* gastritis which is a known preneoplastic condition, is a good model to study the response of stem cells to chronic injury and mutagenesis[13-15]. A recent study has shown a direct interaction between *H. pylori* organisms and gastric stem cells[15].

We have recently demonstrated the combined effects of aging and carcinogen treatment on the colon CSCs in a rat model[16,17]. In this model, carcinogen treated rats had more dramatic increase in CSCs if they were also aged. Based on these and other relevant observations[1,9,17-21], we hypothesize that, aging and chronic inflammation are two parallel events leading to an increased incidence of cancers in the gastrointestinal tract, including colon and gastric cancers. We further hypothesize that the initiating factor in this scenario is the alteration of the CSC population in the normal appearing mucosa.

To test our hypothesis that combination of the effects of aging and inflammation on CSCs exacerbates cancer development, we made an attempt to identify gastric CSCs by using immunohistochemical (IHC) markers in young and old rat gastric mucosa samples. We then expanded our studies to human gastric mucosa with various degrees of *H. pylori* induced inflammation in order to show the alterations in CSC compartment during the course of *H. pylori*- induced disease.

MATERIALS AND METHODS

Animals

Male Fischer-344 rats, aged 4-6 (young) or 22-24 mo (old) were purchased from the National Institute on Aging (Bethesda, MD). All procedures were performed according to the standards for use of laboratory animals established by the Institute of Laboratory Animal Resources, National academy of Sciences, and were approved by the Animal Investigation Committee at Wayne State University School of Medicine. The details of animal handling have been previously published[16,20].

Human gastric tissues

Formalin fixed-paraffin embedded gastric tissue samples representing normal/uninfected mucosa (n = 10), helicobacter pylori gastritis (n = 12), helicobacter pylori gastritis with intestinal metaplasia (n = 10), dysplasia (n = 6) and gastric cancer (n = 12) were retrieved from the Pathology archives of John D. Dingell VA Medical Center, Detroit MI. The diagnoses were confirmed by three pathologists who are co-authors of this study. The study was approved by the IRB committee of Wayne State University, and the R&D committee of John D. Dingell VA Medical Center.

The mean age of the patients was 46 ± 6 (SD). They were all male, reflecting the population profile of the hospital. The difference of age between the control and the inflamed mucosa samples was not statistically significant (not shown).

Immunohistochemistry

The antibodies utilized for immunohistochemical stains

Table 1 Staining scores for stem cell markers in human gastric mucosa and rat gastric mucosa

	n	ALDH	CD166	LGR5
Normal	10	1-2	0	1-2
H. pylori without IM	12	5-6[a]	3-4[a]	3-4[a]
H. pylori with IM	10	7-8[a]	5-6[a]	5-6[a]
Gastric adenocarcinoma	12	85%	75%	70%
4-mo-old rat	6	1-2	1-2	0
24-mo-old rat	6	3-4	5-6[a]	3-4[a]

The numbers are cells expressing the marker per a crypt counted. We grouped the cell counts as 0; 1-2; 3-4; 5-6; 7-8; *etc*. For evaluation of gastric adenocarcinoma, we expressed the percentage of cells staining with the related marker. [a]$P < 0.05$ *vs* normal human gastric mucosa or 4-mo-old rat gastric mucosa. Gastric adenocarcinoma was not included in statistical analysis.

were LGR5 (dilution at 1:200, ABGENT, San Diego CA), CD166 (dilution at 1:200 RD systems, Minneapolis MN), ALDH1 (dilution at 1:100 BD Biosciences, San Jose CA) and B-catenin (SCBT, Dallas TX at 1:100 dilution).

Immunohistochemistry was performed according to our standard protocol[8,13,19]. Briefly, the paraffin blocks of the fixed colon tissues were cut into 5 μm sections. The slides were deparaffinized. For antigen retrieval, tissues were microwaved for 15 min in Citrate pH = 6.0 buffer, then allowed to cool to room temperature. Endogenous peroxide was quenched by incubation of the sections with 3% hydrogen peroxide. Non specific binding was blocked application of 5% horse serum. Primary antibodies were applied overnight at 4 ℃ and antibody detection was completed utilizing the Vecstatin Elite ABC system detection kit from Vector (Burlingame CA). AEC was used as chromogen.

We defined positivity in normal and *H. pylori* cases as membranous and cytoplasmic staining in number of cells per gland (CPG). For cancer cases we used percentage of tumor cells to define positivity.

Immunofluorescence double labeling of B-catenin and LGR5

We have performed double labeling for B-catenin and Lgr5 on sections from rat gastric mucosa and human gastric epithelium by using immunofluorescent secondary antibodies to demonstrate the co-expression of these markers. For B-catenin primary antibody (Santa Cruz BT) anti-mouse IgG TRITC (Sigma, St Louis MO) secondary antibody was used. For LGR5 (ABGENT) antibody, anti-rabbit IgG FITC (Sigma) antibody was used. The slides were evaluated by a fluorescent microscope with green and red filters. Gastric cancer specimens were used as positive controls. For negative controls, we omitted the primary antibody, and applied only secondary antibody.

Statistical analysis

For statistical analysis we assessed the CSC expression as low *vs* high expression, with 0 and 1-2 CSC considered as low expression and ⩾ 3-4 CSC as high expression. This cut off value was based on the observation that in normal mucosa we rarely encountered more than 1-2 CSC per gland counted. Statistical significance was assessed by χ^2 test.

RESULTS

Rat gastric mucosa

In the 4 mo old (young) rats ALDH1, CD166 and LGR5 staining were rare events. Very few cells in the isthmic region of the fundic mucosa demonstrated cytoplasmic staining for the three markers (Figure 1). The results are summarized in Table 1.

The expression of LGR5 and CD166 was significantly increased in the 24 mo old rats (Figure 1 and Table 1). The staining location was still in the isthmus region. The B-catenin staining was limited to few stem cells and was membranous in the young rat mucosa and cytoplasmic in the aged mucosa (Figure 1).

B-catenin LGR5 double labeling

We also investigated the expression of B-catenin and LGR5 in a double immunofluorescent labeling study. B-catenin is normally expressed in the cell membrane in the inactive state. Activated B-catenin pathway can be detected by nuclear or cytoplasmic staining. We therefore investigated the status of B-catenin signaling in the LGR5 expressing putative stem cells. As shown in Figure 2A, a rare cell with LGR5 expression (green signal) also revealed cytoplasmic B-catenin immunoreactivity (red signal).

Human gastric mucosa

We first investigated the expression of CSC markers in normal mucosa which included both antral and fundic mucosa. The staining of the cells was localized to the isthmic region of the fundic mucosa (Figure 3). In antrum, the staining was present in the base of the glands.

In *H. pylori* infected gastric mucosa, the expression of CSC markers was significantly increased for all three markers examined (Table 1 and Figure 3).

In the *H. pylori* infected gastric mucosa with intestinal metaplasia and also in gastric cancer, the level of staining was also significantly increased (Table 1).

In addition, we performed a double labeling immunofluorescent staining for LGR5 and B-catenin in *H. pylori* infected gastric mucosa. We observed that in the LGR5 expressing cells in the gastric mucosa; the expression of B-catenin is cytoplasmic, implying an activation of B-catenin signaling (Figure 2B).

DISCUSSION

In this study we demonstrated an age-related in CSCs in normal appearing gastric mucosa with activated WNT signaling. In addition, we have shown that, gastric infec-

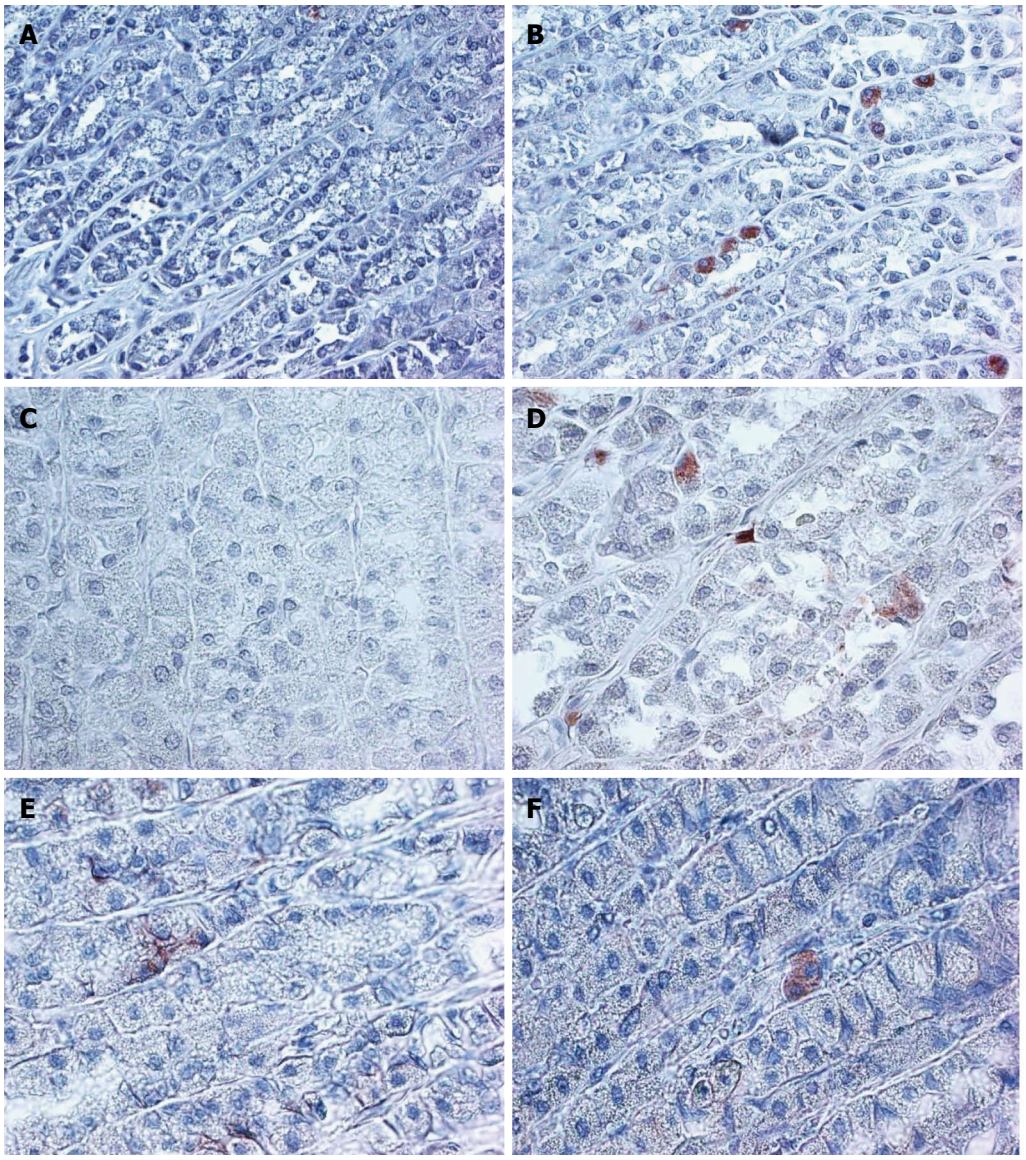

Figure 1 Higher expression of B-catenin, CD166, and LGR5 in normal aged rat gastric mucosa, compared to young rat normal gastric mucosa is demonstrated. × 200 magnification. A: 4 m CD166; B: 24 m CD166; C: 4 m LGR5; D: 24 m LGR5; E: 4 m B-catenin; F: 24 m B-catenin.

tion by *H. pylori* induces an increase in CSC population in the gastric mucosa. Based on our observations we believe that aging and chronic inflammation with *H. pylori* are two significant factors that overlap and presumably exacerbate each other in gastric carcinogenesis.

Studies of the murine gastrointestinal tract have shown that cells from old mice at or near the position of the stem cells within the crypts of Lieberkuhn are more susceptible to apoptosis under stress[18] and exhibit reduced regenerative potential despite an age dependent increase in the number of crypt cells. Similar age related decline in functional properties of stem cells have been shown in other tissues particularly in hematopoietic stem cells[9]. It is very likely that the increase in the number of stem cells is a compensatory event to replenish the destroyed cells in the target tissue and is a reflection of the decreased functional capacity of these cells. This situation is analogous to myelodysplastic syndrome, which is commonly present in the elderly and is characterized by a hypercellular bone marrow despite a peripheral cytopenia.

Stem cells are subject to the similar array of insults as somatic cells and are therefore susceptible to genetic damage. The accumulating damage, unlike that of somatic cells is propagated to the daughter cells and to downstream lineages through the process of self renewal and differentiation. The nature of accumulating mutations and genetic damage determines the fate of the CSCs. The outcome could be senescence, apoptosis, or transformation[9].

In this context, the survival pathways utilized by the stem cells are very critical in their maintenance and possibly transformation. The important signaling pathways involved in gastric CSCs survival include the Wnt/B-catenin, sonic hedgehog (shh), Notch, and fibroblastic growth factor/bone morphogenic protein (FGF/BMP)

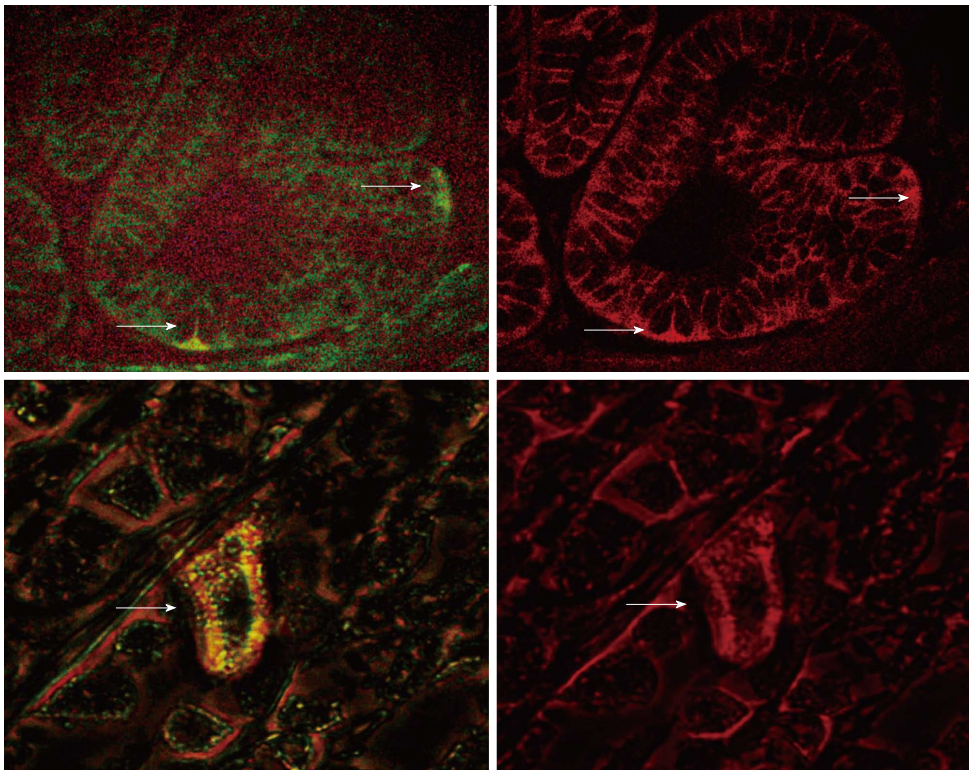

Figure 2 Double labeling for LGR5 (green, left panel) and B-catenin (red, right panel) in aged rat gastric mucosa (Lower Panel, × 600 magnification), *H. pylori* infected human gastric mucosa (Upper Panel, × 400 magnification) shows cytoplasmic localization of B-catenin in an LGR5 expressing cell.

pathways[8,9,12,22].

Wnt signaling pathway releases B-catenin from the AXIN/GSK3b degradation complex. Activation of the Wnt signaling results in the translocation of B-catenin from the cell membrane to the cytoplasm and subsequent translocation to the nucleus. Accumulation of B-catenin in the nucleus results in the transcriptional activation of target genes that play critical roles in regulating cell proliferation[22].

H. pylori infection results in the activation of the stem cell signaling networks such as Wnt, Notch, FGF/BMP, and Hh/SHH oncogenic signaling pathways[2,8,22]. In a previous study, *H. pylori* infection has been shown to be associated with an increased expression of CD44 in gastric mucosa[10]. We also investigated CD44 in the gastric mucosa and found that CD44 expression is markedly increased in *H. pylori* infected mucosa (data not shown).

LGR5 is an orphan G-protein coupled receptor and Wnt target gene, and is a putative marker for gastrointestinal stem cells. Barker *et al*[3] first identified a subpopulation of Lgr5+ stem cells at the base of the crypts in mouse small intestine and colon. Since then, several studies have confirmed the utility of Lgr5 as a putative stem cell/progenitor marker[3-7]. Our studies highlight an Lgr5 positive population in normal human fundic epithelium, localized to the isthmic region, in concordance with the anatomic localization of the progenitor cells. In addition, we demonstrate that Lgr5 expressing cells are increased during aging and in response to *H. pylori* infection.

Our double labeling studies demonstrate that B-catenin is relocated to cytoplasm in the stem cells in aging and *H. pylori* infection. Wnt signaling may preferentially influence the expansion of progenitor cells in the gastrointestinal system and may be the driving force behind the early increase in CSCs in the colon and stomach. However, we do not know whether the activation of B-Catenin is entirely normal or an early phase of neoplastic transformation. Since WNT signaling can support both the normal and CSCs renewal and maintenance, it is a potential target for therapeutic interventions or preventative measures.

Our current findings are very similar to our previous data from colonic mucosa of aged and carcinogen exposed rats[14,16]. We propose that in gastrointestinal cancers, aging and chronic inflammation leading to cytokine activation is a critical factor. The increase in CSCs is probably one of the early events in this process. Further studies are needed to directly observe the link between increase in CSCs and acquisition of cancer phenotype.

The finding of increase in CSCs in otherwise normal appearing mucosa has ramifications for diagnostic, prognostic, preventive, and therapeutic approaches to the gastrointestinal cancers. CSC markers can be used in gastric biopsies from patients with atrophic gastritis and intestinal metaplasia to see the status of CSC population. This can be used as a surrogate for increased risk for cancer. In addition, targeted therapies can be designed to specifically attack the stem cell population in cancers, and response to treatment can be monitored by observ-

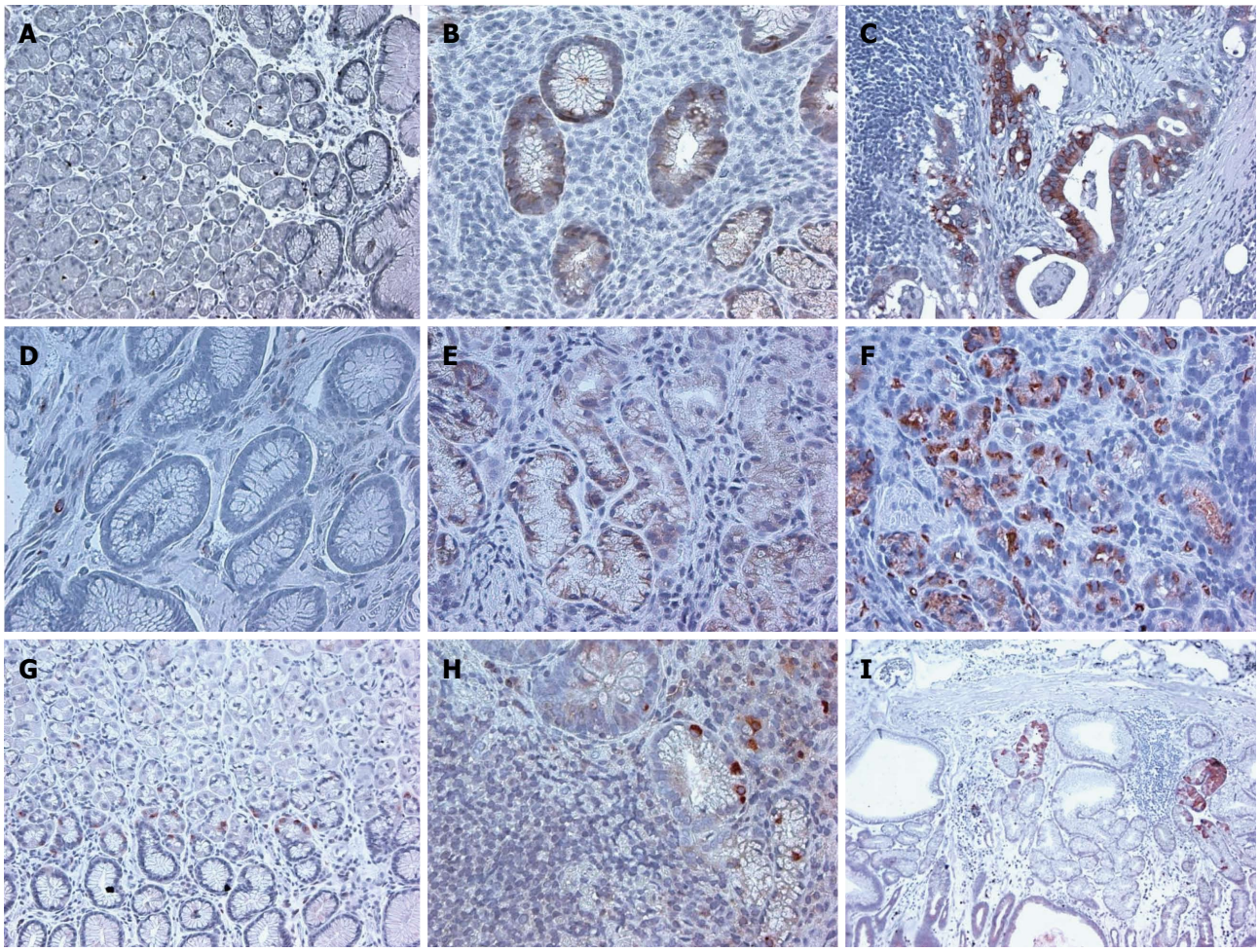

Figure 3 Immunohistochemical staining of stem cell markers ALDH1, CD166, LGR5 in human normal gastric mucosa, gastric mucosa with *H. pylori* gastritis, and gastric adenocarcinoma, demonstrates increased expression of each of the markers over the normal controls. × 200 magnification. A: ALDH1 Normal; B: ALDH1 HP; C: ALDH1 CA; D: CD166 Normal; E: CD166 HP; F: CD166 CA; G: LGR5 Normal; H: LGR5 HP; I: LGR5 CA.

ing the changes in the stem cell population.

COMMENTS

Background

Aging and chronic inflammation are two factors associated with gastric cancer. There is evidence suggesting a link between stem cells in gastric mucosa and increased risk for cancer. Aging and chronic inflammation may cause alterations in stem cells thus causing cancer.

Research frontiers

Cancer stem cells can be detected by using specific markers and demonstrated by immunohistochemistry. This approach allows the authors to demonstrate changes in cancer stem cells associated with aging and inflammation.

Innovations and breakthroughs

In this study the authors demonstrated that aging and chronic inflammation are associated with an increased stem cell population in gastric mucosa.

Applications

Gastric cancer stem cell markers can be utilized as prognostic markers or can be used to monitor response to treatment. They can also help the authors understand tumor pathobiology.

Terminology

Cancer stem cells are thought to be normal resident stem cells or specialized cells which acquire cancer initiating properties. Cancer stem cell hypothesis assumes that cancers arise by alterations in cancer initiating subpopulations of cells in a given tissue.

Peer review

The reviewers think that the study provides data identifying and quantifying stem cells in gastric mucosa of rats and humans. According to their data the number of stem cells is increased by chronic inflammation and aging.

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SYSTEMATIC REVIEWS

Oxidative and nitrosative stress enzymes in relation to nitrotyrosine in *Helicobacter pylori*-infected humans

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Author contributions: Elfvin A and Edebo A performed all the gastroscopies; Elfvin A, Hallersund P and Casselbrant A performed the laboratory work, and analysis; Elfvin A and Fändriks L designed the study; Fändriks L coordinated the study and provided financial support; Elfvin A and Hallersund P wrote the manuscript; all authors were involved in editing the manuscript.
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Abstract

AIM: To compare a possible relation between *Helicobacter pylori* (*H. pylori*) and the oxygen- and nitrogen radical system in humans.

METHODS: Mechanisms for *H. pylori* to interfere with the oxygen and nitrogen radical system is of great importance for understanding of the *H. pylori* persistence and pathogenesis. Biopsies were obtained from the gastric wall of 21 individuals. Ongoing infection with *H. pylori* was detected using direct analyze from the biopsies using campylobacter-like organism test (CLO-test) and/or by using ^{14}C-urea breath test. The individuals were divided in a negative *H. pylori* and a positive *H. pylori* group. Expression in the gastric mucosa of inducible nitric oxide syntase (iNOS), nicotinamide adenine dinucleotide phosphate-oxidase (NADPH-oxidase) myeloperoxidase (MPO), and nitrotyrosine were assessed by Western blotting.

RESULTS: The individuals who undervent gastroscopy were divided in a *H. pylori* neg. [*n* = 13, m/f = 7/6, age (mean) = 39] and a *H. pylori* pos. group [*n*= 8, m/f = 5/3, age (mean) = 53]. Using western blot analysis iNOS was detected as a 130 kDa band. The iNOS expression was upregulated in the antrum of H. pylori infected individuals in comparison to the controls, mean ± SD being 12.6 ± 2.4 *vs* 8.3 ± 3.1, $P < 0.01$. There was a markedly upregulated expression of MPO in the antrum of *H. pylori* infected individuals in comparison to the control group without infection. In several of non-infected controls it was not possible to detect any MPO expression at all, whereas the expression was high in all the infected subjects, mean ± SD being 5.1 ± 3.4 *vs* 2.1 ± 1.9, $P < 0.05$. The NADPH-oxidase expression was analysed by detecting the NADPH-oxidase subunit p47-phox expression. P47-phox was detected as a 47 kDa band using Western blot, and showed a significantly higher expression of p47-phox in the antrum of the *H. pylori* infected individuals compared to the controls, mean ± SD being 3.1 ± 2.2 *vs* 0.3 ± 0.2, $P < 0.01$. Regarding nitrotyrosine formation, Western blot did not show any significant increase or decrease compared to controls, 7.0 ± 0.9 *vs* 6.9 ± 1.1, not significant.

CONCLUSION: iNOS, MPO and NADPH-oxidase was up-regulated among *H. pylori* infected. Regarding nitrotyrosine no difference was found. This support an *H. pylori* related inhibition of radical formation.

Key words: *Helicobacter pylori*; Radical; Myeloperoxidase; Nicotinamide adenine dinucleotide phosphate; Nitrotyrosine; Gastric

Core tip: The present project was performed to compare a possible relation between *Helicobacter pylori* (*H. pylori*) and the oxygen- and nitrogen radical system in humans. Expression of inducible nitric oxide syntase, myeloperoxidase and nicotinamide adenine dinucleotide phosphate-oxidase was upregulted in the antrum of the group with *H. pylori* infection. Regarding nitrotyrosine formation, Western blot did not show any significant increase or decrease compared to controls. The present study illustrates the complex picture of the oxidative stress in relation to *H. pylori* infection. The present study supports the theory of an *H. pylori* related inhibition of the enzymes involved in the oxy- and/or nitro-radical formation pathway.

Elfvin A, Edebo A, Hallersund P, Casselbrant A, Fändriks L. Oxidative and nitrosative stress enzymes in relation to nitrotyrosine in *Helicobacter pylori*-infected humans. *World J Gastrointest Pathophysiol* 2014; 5(3): 373-379 Available from: URL: http://www.wjgnet.com/2150-5330/full/v5/i3/373.htm DOI: http://dx.doi.org/10.4291/wjgp.v5.i3.373

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a pathogen colonizing the human gastric mucosa playing a significant role in the development of gastric ulcer, gastritis, and gastric cancer[1] Until recently there was insufficient knowledge about how *H. pylori* could avoid being eliminated by the acute host defence and establish a chronic infection in the gastric mucosa of humans. Recent studies have shown that *H. pylori* interferes with reactive oxygen species (ROS) such as superoxide anion (O_2^-) that is of importance in the elimination of invading microorganisms[2,3]. At the same time reactive nitrogen intermediates such as nitric oxide (NO) represent another class of oxidants. NO can be formed as a nitrogenous product of nitric oxide synthase (NOS). Peroxynitrite, formed by NO and O_2^-, is a very powerful oxidant. It is unstable with dimensions related to the hydroxyl radical[4]. In neutrophils and macrophages large amounts of reactive oxygen and nitrogen species are presented to the invading microorganism. Neutrophils phagocytose bacteria into the intracellular phagosome, where an eruption of reactive species results in bacterial destruction. During successful conditions the bacteria is eliminated and there is no extracellular oxidant generation[5].

However *H. pylori* persist in the gastric mucosa, causing a chronic infection that increases the risk for pathological changes such as adenocarcinoma. Therefore the mechanisms for *H. pylori* to interfere with the oxygen and nitrogen radical system is of great importance for understanding the persistence and pathogenesis of *H. pylori*.

We and others have pointed out the association between *H. pylori* infection and an increased mucosal expression of iNOS both in humans and in mongolian gerbils[6-8]. Despite what one could expect, the juxtamucosal level of nitric oxide (NO) is lower in the infected than in the uninfected stomach[7,8]. We have shown that there is an inhibition of nitrotyrosine expression, being a reflection of the formation of peroxynitrite, in *H. pylori* infected Mongolian gerbils despite upregulated formation of both myeloperoxidase (MPO) and inducible nitric oxide synthase (iNOS)[6]. Results from *in vivo* registration of NO and hydrogen peroxide (H_2O_2) on Mongolian gerbils substantiates the fact infection with *H. pylori* reduces levels of NO[9]. It is recently suggested that specific proteins contained by *H.pylori* enables the pathogen to cope with the damaging effects of NO. These systems are suggested to be a part in the microbial protection against nitrosative stress[10]. Several traditional anti-inflammatory drugs have been shown to have an effect on epithelial cells infected by *H.pylori* by inhibiting the induction of iNOS by suppressing the activation of NADPH oxidase[11] .

The present project was performed to further compare a possible relation between *H. pylori* and the oxygen- and nitrogen radical system in humans.

Special interest was on the suspected upregulation on the enzymatic oxy- and nitro radical systems, and if this would result in an increased radical formation. To evaluate activity of peroxynitrite, expression of nitrotyrosine was used as an indicator of radical formation.

MATERIALS AND METHODS

Ethics approval

Approval was obtained from the Research Ethics Committee at Sahlgrenska Academy, Gothenburg University and from the Gothenburg Regional Ethical Review Board.

Study groups

Gastric biopsies were obtained from the antral wall of 21 individuals. The individuals were divided in a *H. pylori* neg. [n = 13, m/f = 7/6, age (mean) = 39] and a *H. pylori* pos. group [n = 8, m/f = 5/3, age (mean) = 53]. Gastro-esophageal reflux (GER) was diagnosed in one subject in the *H. pylori* pos group and in four subjects in the *H. pylori* neg group. Ulcer in the duodenum was found in two individuals in the *H. pylori* pos group.

Diagnostic procedures

Ongoing infection with *H. pylori* was detected using direct analyze from the biopsies using campylobacter-like organism test (CLO-test) and/or by using ^{14}C-urea breath test[12].

Western blot

Biopsies were collected during gastroscopy. The samples were snap-frozen in nitrogenum liquidum and kept for further analysis at -70 ℃. Sonication (Sonifier 450/250, Branson Ultrasonics Co. Danbury, United States) or homogenization (Polytron, PT-MR 2100, Kinematica) was performed of all samples at 2 ℃ in a PE-buffer (10

mmol/L potassium Phosphate buffer, pH 6.8, and 1 mmol/L EDTA) containing CHAPS {3-[(3-cholamidopropyl) dimethyl-ammanio] 1-propanesulfonate}, aprotinin (1 μg/mL), leupeptin (10 μg/ mL), pepstatin (10 μg/mL) and Pefablock (1 mg/mL) (Boeringer Mannheim, Mannheim, Germany). All samples were centrifugated at 10.000 *g* for 10 min at 4 ℃. Analysation was performed of the supernatant for protein content using the method of Bradford[12] and then kept at -70 ℃ for future analysis. Samples were diluted in SDS-buffer and heated at 70 ℃ for 10 min before they were loaded on a NuPage 10% BisTris gel (Invitrogen, Carlsbad, CA, United States). One lane of each gel was loaded with prestained molecular weight standards (SeeBlue™, Invitrogen, Carlsbad, CA, United States). Following the electrophoresis the proteins were transferred to a polyvinyldifluoride membrane (Amersham, Buckinghamshire, United Kingdom) which was incubated with antibodies directed against iNOS, MPO, NADPH-oxidase or nitrotyrosine containing proteins. For identifying iNOS the NOS2 (H-174) sc-8310 (Santa Cruz Biotechnology inc) antibody was used. It is a rabbit polyclonal antibody raised against a recombinant protein corresponding to amino acids 2-175 mapping at the amino terminus of iNOS of human origin. Lack of cross-reaction with nNOS or eNOS was reported by manufacturer. Antibody Anti-myeloperoxidase 07-496 lot 24587 (Upstate, Lake Placid, NY, United States) was used for detecting MPO. This is a rabbit antibody that recognizes MPO subunits at 12 and 60 kDa. In the present study the 60 kDa band was used for quantification of the protein. Anti-nitrotyrosine rabbit immunoaffinity purified IgG catalog 06-284, lot 26427 (Upstate, Lake Placid, NY, United States) was used to assess nitrated proteins. For identifying NADPH-oxidase the p47-phox (H-195) sc 14015 (Santa Cruz Biotechnology inc.) was used. This is a rabbit polyclonal antibody raised against amino acids 196-390 of p47-phox of human origin. P47-phox is required for activation of NADPH-oxidase in neutrophils and other phagocytic cells. During activation of NADPH-oxidase, p47-phox migrate to the plasma membrane where it associates with other subunits to form the active complex. Goat anti-rabbit antibodies were used to identify immunoreactive proteins by chemiluminescence [iNOS, NADPH-oxidase (p47-phox) and nitrotyrosine; goat-anti rabbit sc 2007(Santa Cruz, CA, United States)] [MPO; IgG 12-448 (Upstate Lake Placid, NY, United States)]. CDP-Star (Tropix, Bedford, MA, United States) was used as a substrate. Images were captured by a LAS-100 cooled CCD-camera (Fujifilm, Tokyo, Japan) and semi-quantification was performed using the soft ware Gauge 3.3 (Fujifilm, Tokyo, Japan). As positive controls, to confirm the identity of the protein, RAW 264.7 (sc 2212, Santa Cruz Biotech) was used for iNOS, HL60 was used for MPO and NADPH-oxidase (p47-phox).For nitrotyrosine the immunoblotting control (12-354, Upstate) was used.

Statistical analysis

Statistical analysis was performed using non parametric Mann-Whitney *U*-test. *P*-values of < 0.05 were regarded as being of statistical significance.

RESULTS

Inducible nitric oxide synthase

Using western blot analysis iNOS was detected as a 130 kDa band. The iNOS expression was upregulated in the antrum of *H. pylori* infected individuals in comparison to the control group without infection as shown in Figure 1A, mean ± SD being 12.6 ± 2.4 *vs* 8.3 ± 3.1, $P < 0.01$. Western blot detecting iNOS with a band at 130 kDa in RAW 264.7 (pos contr.), and in human antral mucosa retrieved from *H. pylori* pos. and *H. pylori* neg. volunteers during endoscopy is shown in Figure 2.

Myeloperoxidase

As shown in Figure 1B, MPO expression was markedly upregulated in the antrum of the *H. pylori* infected individuals in comparison to the control group without infection, mean ± SD being 5.1 ± 3.4 *vs* 2.1 ± 1.9, $P < 0.05$. In several of the non-infected controls it was not possible to detect any MPO expression at all, whereas the expression was high in all the infected subjects. Western blot of the MPO positive 60 kDa band in the positive HL60 control and in gastric mucosal specimens of *H. pylori* pos. and *H. pylori* neg. volunteers is shown in Figure 2.

NADPH-oxidase

The expression of NADPH-oxidase was analysed by detecting the NADPH-oxidase subunit p47-phox expression. P47-phox was detected as a 47 kDa band using Western blot. Figure 1C shows a significantly higher expression of p47-phox in the the antrum of *H. pylori* infected individuals in comparison to the control group without infection, mean ± SD being 3.1 ± 2.2 *vs* 0.3 ± 0.2, $P < 0.01$. P47-phox was low in all non-infected controls. In the *H. pylori* infected subjects there was a large spreading of the p47-phox expression. A typical Western blot result is shown in Figure 2.

Nitrotyrosine

Western blot analysis did not show any significant increase nor decrease in nitrotyrosine expression the antrum of *H. pylori* infected individuals in comparison to the control group without infection, 7.0 ± 0.9 *vs* 6.9 ± 1.1, not significant (Figure 3). Regarding Western blot representing Nitrotyrosine, several bands of Nitrated proteins could be analysed. Shown in the Figure 2 is a typical 66 kDa band in the positive control and in *H.pylori* pos. and *H. pylori* neg. subjects.

DISCUSSION

The findings of the present investigation can confirm that *H. pylori* infection in humans is related to an up regulation of the expression of MPO, iNOS and NADPH-

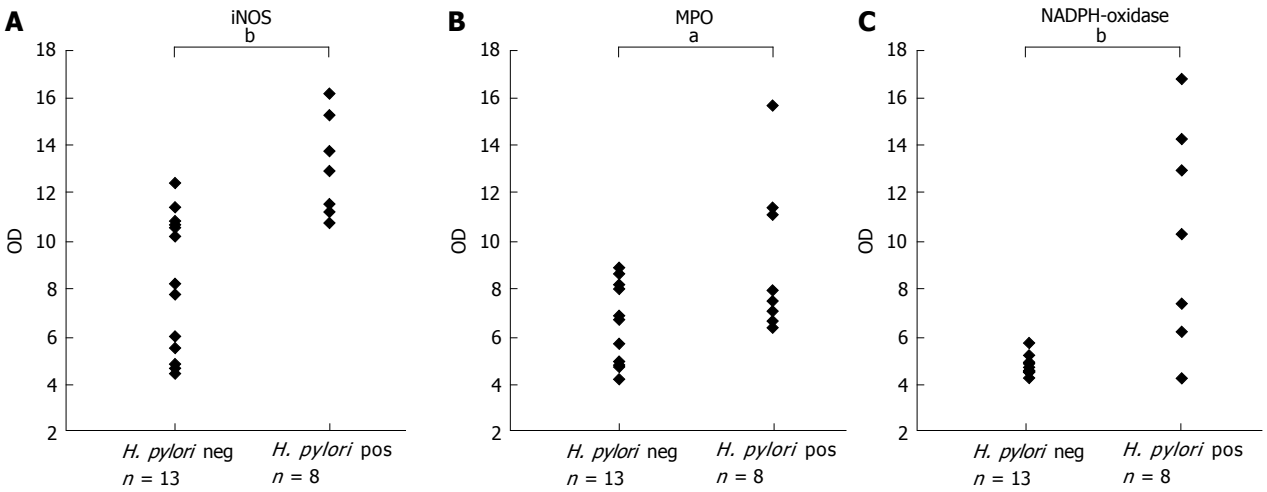

Figure 1 Scatter-plot demonstrating the result of Western blot inducible nitric oxide synthase, myeloperoxidase and nicotinamide adenine dinucleotide phosphate-oxidase protein expression in biopsies from the anrum of the *Helicobacter pylori* neg (*n* = 13) and *Helicobacter pylori* pos (*n* = 8) groups. A: Inducible nitric oxide synthase (iNOS), $^{b}P < 0.01$; B: Myeloperoxidase (MPO), $^{a}P < 0.05$; C: Nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase subunit p47-phox in biopsies from the antrum of the *H. pylori* neg. (*n* = 13) and *H. pylori* pos. (*n* = 8) groups, $^{b}P < 0.01$, OD: Optical density.

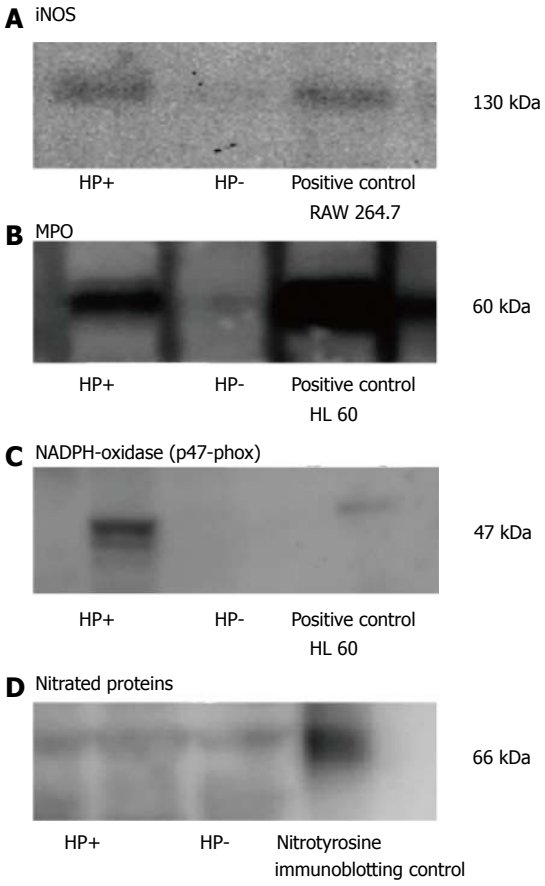

Figure 2 Western blot. A: Western blot detecting inducible nitric oxide syntase (iNOS) with a band at 130 kDa in RAW 264.7 (pos contr.), and in human antral mucosa retrieved from *Helicobacter pylori* (*H. pylori*) pos. and *H. pylori* neg. volunteers during endoscopy; B: Western blot of the MPO positive 60 kDa band in the positive HL60 control and in gastric mucosal specimens of *H.pylori* pos. and *H.pylori* neg. volunteers; C: Western blot of p47-phox, representing NADPH-oxidase with a band at 47 kDa (pos. contr.) HL60, and in HP+ and HP- samples; D: Regarding Western blot representing Nitrotyrosine, several bands of Nitrated proteins could be analysed. Shown in the figure is a typical 66 kDa band in the pos. control and in *H.pylori* pos. and *H.pylori* neg. subjects.

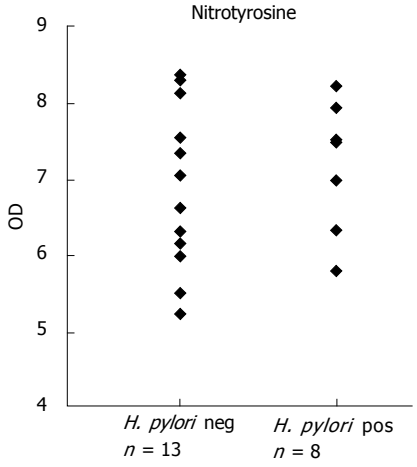

Figure 3 Nitrotyrosine. Scatter-plot demonstrating the result of Western blot analysis of nitrotyrosine formation in biopsies from the antrum of the *Helicobacter pylori* (*H. pylori*) neg. (*n* = 13) and *H. pylori* pos. (*n* = 8) groups. There were no significant changes between groups. OD: Optical density.

oxidase in the human gastric mucosa. Furthermore the study shows that there are no significant changes in levels of proteins containing nitrotyrosine compared to non-infected subjects following this up-regulation. This finding confirms the results from our studies on Mongolian gerbils, and supports the theory of an *H. pylori* related inhibition of radical formation[6,9].

Studying the early stages of *H. pylori* infecting the stomach is important for understanding the evolution of pathology such as carcinogenesis. Using an animal model makes it possible to assess different stages of pathological development in an experimental setting. However it is important to evaluate the experimental findings in a human population before making any conclusions regarding *H. pylori* infection in human gastric mucosa.

The initial host reaction to the *H. pylori* infection is

the same as to any bacterial infection: Phagocytic neutrophils and monocytes are recruited to the infected tissue and consume oxygen that is converted to O_2^- by NADPH-oxidase, and then dismutated to H_2O_2[13]. Activation of neutrophils results in the release of MPO, which catalyzes the oxidation of electron donors by H_2O_2[14]. A complex is formed that is responsible for the production of powerful oxidants with potential to react with a large variety of substances[15,16]. For example, MPO-H_2O_2 reacts with chloride to form hypochlorus acid and subsequently the oxidative chloramines are formed. The MPO-H_2O_2-chloride system is responsible for many biological effects, both beneficial and negative for the host[17].

In general, inflammation results in invading epithelial cells and macrophages leading to a marked expression of iNOS and resulting in generation of NO[17].

Several studies have described an increase in iNOS production following *H. pylori* infection in both humans and animal models[6-8,18-20]. Some have suggested that the up-regulated iNOS production following *H. pylori* infection would lead to an increase in NO production which could result in the increase of DNA damage and apoptosis[18-21]. It has been suggested that classification of iNOS expression in the gastric mucosa could be used clinically to identify patients with a high risk for gastric cancer[22]. The host will try to terminate the infection by activating the mucosal generation of the oxy- and nitro-radical forming enzymes the resulting in formation of the cytotoxic peroxynitrite. In the extracellular space NO released from macrophages can eliminate *H. pylori*[23]. An effective increase of production of NO and oxy-radicals would lead to eradication of the bacteria. However *H. pylori* persist in the host, causing a chronic inflammatory reaction that instead in the long run may be deleterious to the host. The fact that *H. pylori* survives in this hostile environment despite up regulation of iNOS suggests that the pathogen has developed strategies to avoid NO-dependent eradication. An increasing number of studies have reported about the complexity of the *H. pylori* response to oxidative and nitrogen stress[24,25].

H. pylori may also have a direct effect on reduction in gastric mucosal blood flow by inhibiting NO production by iNOS and thereby reducing the vasodilatory and mast cell stabilizing effect of NO[26].

We have by use of electrochemical microelectrodes *in vivo* confirmed reduction of intraluminal NO in Mongolian gerbils following infection with *H.pylori*[9]. Reduced levels of NO could be explained by inhibition of iNOS activity[27]. Helicobacter produced arginase has been proposed as one of the ways for *H. pylori* to inhibit NO production[23,25]. *H. pylori* may also produce asymmetrical dimethyl arginine (ADMA) that can block iNOS by competitive inhibition. ADMA is a methylated form of arginine that has been found to be significantly up-regulated in the human antrum of *H. pylori* positive individuals[8,28]. Another explanation for reduced NO levels could be scavenge of NO by reacting with reactive oxygen species (ROS)[7,18]. A result of this reaction would be an increase in the production of peroxynitrite, and resulting in increased levels of nitrotyrosine. Thus nitrotyrosine can be used to indicate peroxinitrite activity over time. The present investigation as well as previous studies on *H. pylori* infection in Mongolian gerbil demonstrates a significant up regulation of the formation of iNOS and MPO, but no significant changes in the levels of nitrotyrosine[6]. These findings strongly support the theory supports the theory of an *H. pylori* related inhibition of radical formation at an enzymatic level of NO generation.

The present study does not provide data on if *H. pylori* also inhibit the oxy-radical forming enzymes. Oxidative stress could potentially have a negative effect on the capacity of *H. pylori* to infest the human stomach. However it is shown that *H. pylori* produces a number of antioxidative proteins, the most described ones being bacterial produced superoxide dismutase (SOD)[29]. SOD production is described as being of importance for *H. pylori* being able to grow and survive in a situation with oxidative stress, and is regarded as a factor being of importance for of the microbial colonization of the stomach. Catalase and arginase are other examples of antioxidant proteins produced by *H. pylori* that might contribute to bacterial survival under conditions of oxidative stress[23,30,31].

Taken together the present study illustrates the complex picture of the oxidative stress response to *H. pylori* infection. The nitro- and oxy-radical formation systems are up-regulated following infection and inflammation. This up-regulation is to be regarded as an attempt from the host to eradicate the bacteria. However, long standing up-regulation of the reactive oxygen- and nitrogen species will also lead to tissue damage and a risk of carcinogenesis. This study supports the theory supports the theory of an *H. pylori* related inhibition the. The mechanisms behind how the bacteria and the impotent host defence act to induce DNA- and tissue damaging effects need to be further explored.

The results suggest that there is a relationship between inhibition of formation of ROS and reactive nitrogen species and *H. pylori* being able to survive in the human gastric mucosa.

COMMENTS

Background

Helicobacter pylori (H. pylori) colonization of the mucosal space of the stomach causes a chronic infection resulting in the development of pathological changes such as adenocarcinoma. Mechanisms for *H. pylori* to interfere with the oxygen and nitrogen radical system is of great importance for understanding the persistence and pathogenesis of *H. pylori*.

Research frontiers

Several studies have described an increase in inducible nitric oxide syntase (iNOS) production following *H. pylori* infection in both humans and animal models. An effective increase of production of NO and oxy-radicals would lead to eradication of the bacteria. The fact that *H. pylori* survives in this hostile environment despite up regulation of iNOS suggests that the pathogen has developed strategies to avoid NO-dependent eradication. The findings of the present investigation can confirm that *H. pylori* infection in humans is related to an up regulation of the expression of MPO, iNOS and NADPH-oxidase in the human gastric mucosa. Furthermore the study shows that there are no significant

changes in levels of proteins containing nitrotyrosine compared to non-infected subjects following this up-regulation.

Breakthroughs and innovations

The investigation presented here illustrates the complex picture of the oxidative stress response to *H. pylori* infection. The nitro- and oxy-radical formation systems are up-regulated following infection and inflammation. This up-regulation is to be regarded as an attempt from the host to eradicate the bacteria. However, long standing up-regulation of the reactive oxygen- and nitrogen species will also lead to tissue damage and a risk of carcinogenesis. This study supports the theory of an *H.pylori* related inhibition of formation of reactive oxygen species (ROS) and reactive nitrogen species. The mechanisms behind how the bacteria and the impotent host defence act to induce DNA- and tissue damaging effects need to be further explored.

Applications

By understanding how *H. pylori* manages not to be extinguished in the hostile environment by hindering the formation of reactive oxygen species and reactive nitrogen intermediates we will gain a greater understanding of the mechanisms involved in *H. pylori* related disease.

Terminology

Myeloperoxidase (MPO) is an enzyme of importance in the microbicidal role of phagocytes. iNOS was first identified in macrophages. iNOS is involved in the production of NO, but has also many other functions. Nicotinamide adenine dinucleotide phosphate-oxidase (NADPH-oxidase) is a transmembrane electron transport chain involved in the production of different ROS. Nitrotyrosine can be used as marking the activity of peroxynitrite.

Peer review

In this study the authors demonstrated, in human gastric mucosa of *H.pylori* positive patients, an increase of some enzymes belonging to oxidative stress pathway, while the amount of nitrotyrosine rich proteins did not differ from *H.pylori* negative tissues.

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WJGP 5th Anniversary Special Issues (1): *Helicobacter pylori*

Helicobacter pylori and pancreatic diseases

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Abstract

A possible role for *Helicobacter pylori* (*H. pylori*) infection in pancreatic diseases remains controversial. *H. pylori* infection with antral predomination leading to an increase in pancreatic bicarbonate output and inducing ductal epithelial cell proliferation could contribute to the development of pancreatic cancer *via* complex interactions with the ABO genotype, dietary and smoking habits and N-nitrosamine exposure of the host. Although the individual study data available so far is inconsistent, several meta-analyses have reported an increased risk for pancreatic cancer among *H. pylori* seropositive individuals. It has been suggested that *H. pylori* causes autoimmune pancreatitis due to molecular mimicry between *H. pylori* α-carbonic anhydrase (α-CA) and human CA type Ⅱ, and between *H. pylori* plasminogen-binding protein and human ubiquitin-protein ligase E3 component n-recognin 2, enzymes that are highly expressed in the pancreatic ductal and acinar cells, respectively. Future studies involving large numbers of cases are needed in order to examine the role of *H. pylori* in autoimmune pancreatitis more fully. Considering the worldwide pancreatic cancer burden, as well as the association between autoimmune pancreatitis and other autoimmune conditions, a complete elucidation of the role played by *H. pylori* in the genesis of such conditions could have a substantial impact on healthcare.

Key words: *Helicobacter pylori*; Pancreatic cancer; Pancreatitis; Autoimmune pancreatitis; Molecular mimicry

Core tip: *Helicobacter pylori* (*H. pylori*) infection with antral predomination could contribute to the development of pancreatic cancer through complex interactions with ABO genotypes, dietary and smoking habits and N-nitrosamine exposure of the host. It has been suggested that *H. pylori* causes autoimmune pancreatitis due to molecular mimicry between *H. pylori* α-carbonic anhydrase (α-CA) and human CA type Ⅱ, and between *H. pylori* plasminogen-binding protein and human ubiquitin-protein ligase E3 component n-recognin 2. Considering the worldwide burden of pancreatic diseases, complete elucidation of *H. pylori* role in their genesis could have substantial healthcare impact.

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INTRODUCTION

Helicobacter pylori (*H. pylori*), the ubiquitous bacterium that colonizes the human stomach, has been the subject of increased attention in the last 30 years. It has been sug-

gested that modern humans were infected with *H. pylori* before their migration from Africa over 58000 years ago and that *H. pylori* strains have been intimately associated with their human host populations ever since[1]. Over half the modern human population is infected with *H. pylori*, and its prevalence varies from 60%-90% in Japan, China, Russia and most of Central and Eastern Europe to 30%-40% in Western Europe and the United States[2]. *H. pylori* is proven to be associated with an increased risk for gastric cancer[3], peptic ulcer disease[4] and lymphoma[5]; however, a possible role for *H. pylori* infection in pancreatic disease remains controversial.

Previous studies have examined the association between *H. pylori* infection and diseases of the pancreas, including pancreatic carcinoma[6-12] and autoimmune pancreatitis[13-15], but with inconsistent results. Nevertheless, there is a solid theoretical basis for explaining the potential role for *H. pylori* in the development of these conditions. It has been proposed that *H. pylori* causes autoimmune pancreatitis due to molecular mimicry between *H. pylori* α-carbonic anhydrase (α-CA) and human CA type Ⅱ[14], and it is known that the homologous CA segments contain the binding motif of the HLA molecule DRB1*0405, which confers a risk of developing autoimmune pancreatitis. Furthermore, it has been suggested that *H. pylori* infection contributes to the development of pancreatic cancer *via* complex interactions with the ABO genotype, dietary and smoking habits and N-nitrosamine exposure of the host[16].

Pancreatic cancer is the eighth leading cause of cancer-related deaths worldwide[17] with the five year survival rate as low as 6%[18]. Autoimmune pancreatitis is a relatively novel clinical entity defined as a chronic inflammation of the pancreas due to an autoimmune mechanism[19]. Although autoimmune pancreatitis accounts for a relatively small proportion of chronic pancreatitis cases it can be associated with other autoimmune conditions, suggesting a possible involvement of the entire gastrointestinal system. With this in mind, elucidating the role of *H. pylori* in the development of pancreatic diseases could have a substantial impact on health care.

We have, therefore, conducted a comprehensive literature search in order to summarize the evidence for a role for *H. pylori* in the pathogenesis of pancreatic diseases with particular emphasis on pancreatic cancer and autoimmune pancreatitis.

H. PYLORI AND PANCREATIC CANCER

To date, no study has isolated *H. pylori* DNA in any pancreatic sample[20,21]; however, although *H. pylori* appears not to colonize the pancreas it could have an effect on pancreatic carcinogenesis through pathophysiological action. *H. pylori* shows two different colonization behaviors: one associated with pangastritis leading to hypochlorhydria, atrophic gastritis, gastric ulcer and gastric cancer, and the other associated with antral-predominant gastritis leading to hyperchlorhydria, pyloric and duodenal ulcer and, potentially, pancreatic cancer. Colonization of the antrum by *H. pylori* reduces the number of antral D-cells thus suppressing the production of somatostatin. This, in turn, leads to hyperacidity, which results in an increase in the secretion of secretin and pancreatic bicarbonate output. Secretin has been shown to have a positive effect on murine pancreatic growth as well as DNA synthesis in pancreatic ductal cells[22], and it is possible that induced ductal epithelial cell proliferation could enhance the carcinogenic effect of known carcinogens, such as N-nitrosoamines, in the pancreas, leading to the development of pancreatic cancer.

Although this assumption is hypothetical and needs to be proven there is indirect proof suggesting that *H. pylori* does play a role in pancreatic carcinogenesis. A number of serology-based studies have assessed the association between the presence of anti-*H. pylori* antibodies and pancreatic cancer[6-12]. The first of these, conducted by Raderer *et al*[6], reported a two-fold increase in the risk for pancreatic cancer among *H. pylori*-positive individuals [Odds ratio (OR) = 2.1, 95%CI: 1.09-4.05]. These findings were confirmed in the subsequent Alpha-Tocopherol, β-Carotene Cancer Prevention Study (ATBC Study), a prospective cohort study of male smokers that reported subjects positive for *H. pylori* antibodies or CagA-positive *H. pylori* strains to be at increased risk of developing pancreatic cancer (OR = 1.87, 95%CI: 1.05-3.34; OR = 2.01, 95%CI: 1.09-3.70, respectively)[7].

In contrast, two succeeding studies[8,9], each following patients for 20 years or more, reported no significant association between *H. pylori* infection and pancreatic cancer. In a nested case-control study of 104 pancreatic cancer cases and 262 matched controls, de Martel *et al*[8] selected patients from among 128992 adult subscribers to the Kaiser Permanente Medical Care Program who had been enrolled from 1964 to 1969, and found no association between *H. pylori* (OR = 0.85, 95%CI: 0.49-1.48) or its CagA protein (OR = 0.96, 95%CI: 0.48-1.92) and the subsequent development of pancreatic cancer. In the second study, Lindkvist *et al*[9] conducted a similar analysis on subjects from the Malmö Preventive Project cohort. After analysis of 87 cases and 263 matched controls the researchers reported that *H. pylori* seropositivity was not associated with pancreatic cancer (OR = 1.25, 95%CI: 0.75-2.09). Finally, a case-control study in a Polish population also reported that neither *H. pylori* (OR = 1.27, 95%CI: 0.64-2.61) nor CagA (OR = 0.90, 95%CI: 0.46-1.73) seropositivity were significant risk factors for pancreatic cancer[10].

However, Risch *et al*[11] were the first to suggest that infection with CagA-negative *H. pylori* could be a risk for pancreatic cancer. In a United States population-based case control study, conducted on 373 pancreatic cancer cases and 690 controls, the researchers reported that CagA-negative *H. pylori* seropositivity was a significant risk factor for pancreatic cancer (OR = 1.68, 95%CI: 1.07-2.66), while no significant association was reported for CagA-positive seropositivity (OR = 0.77, 95%CI: 0.52-1.16). Furthermore, the group observed the association between a pancreatic cancer risk and CagA-negative

H. pylori seropositivity only among individuals with a non-O blood type but not among those with O blood type (OR = 2.78, 95%CI: 1.49-5.20; OR = 1.28, 95%CI: 0.62-2.64, respectively), supporting a role for the ABO blood group system in mediating *H. pylori* carcinogenic potential in the pancreas. The same group conducted a similar study on the Chinese population of Shanghai and reported an increased, but not significant, risk of developing pancreatic cancer for CagA-negative *H. pylori* seropositive patients (OR = 1.28, 95%CI: 0.76-2.13)[12]. In addition, CagA-positive seropositivity was shown to protect against pancreatic cancer when compared to *H. pylori* seronegative individuals (OR = 0.68, 95%CI: 0.54-0.84).

Several meta-analyses have attempted to summarize the existing data on the role of *H. pylori* in pancreatic carcinogenesis[16,23,24] including different number of studies based on differences in inclusion criteria. All reported a significant increase in the risk of developing pancreatic cancer among *H. pylori*-positive individuals, with the summary OR ranging from 1.65 (95%CI: 1.30-2.09)[16] to 1.38 (95%CI: 1.22-1.77)[23]. However, none of the meta-analyses reported a significant association between CagA-positive seropositivity and pancreatic cancer[23,24].

Bearing all this data in mind, it could be concluded that the published scientific evidence (although somewhat inconsistent) supports a role for *H. pylori* in the development of pancreatic cancer. The exact mechanism involved in the influence of *H. pylori* on pancreatic carcinogenesis is still unclear and has yet to be explained fully. However, if *H. pylori* is found to increase the risk of developing pancreatic cancer, this could be another reason for targeting *H. pylori* for eradication, especially in individuals with a specific genetic burden, such as a family history of pancreatic cancer.

H. PYLORI AND PANCREATITIS

Although there have been some studies on animal models suggesting a possible role for *H. pylori* infection in acute pancreatitis[25], no author has so far reported a significant association between *H. pylori* infection and acute pancreatitis in humans. Khan *et al*[13] undertook a study of 50 patients with acute alcoholic pancreatitis and 50 alcoholic controls but found no association between *H. pylori* infection and the occurrence of acute pancreatitis.

However, the relationship between *H. pylori* and chronic pancreatitis, and autoimmune chronic pancreatitis in particular, has been the subject of more research. In approximately 60% of cases autoimmune pancreatitis is associated with the presence of other autoimmune diseases such as Sjögren's syndrome, sclerosing extrahepatic cholangitis, primary biliary cirrhosis, autoimmune hepatitis, retroperitoneal fibrosis, salivary gland swelling, inflammatory bowel disease, Hashimoto's thyroiditis and gastric peptic ulceration[26-28]. All of these diseases, including autoimmune pancreatitis itself, are characterized by similar pathohistological findings including fibrotic changes and/or lymphoplasmacytic inflammation. However, to date, no study has isolated *H. pylori* DNA from samples of patients affected with autoimmune pancreatitis[21].

It has been suggested previously that *H. pylori* infection exists as a possible common cause of these conditions acting *via* a mechanism involving the molecular mimicry of host structures[29]. In 2005 Guarneri *et al*[14] reported significant homology between human CA type Ⅱ and *H. pylori* α-CA, an enzyme fundamental for the survival of the bacterium in the gastric environment. As human CA type Ⅱ is expressed in the pancreatic ductal epithelium, *H. pylori* could trigger autoimmune pancreatitis by mimicking the host's CA type Ⅱ protein. Then, in 2009, Frulloni *et al*[15] identified *H. pylori* plasminogen-binding protein (PBP) antibodies in 95% of patients with autoimmune pancreatitis. However, PBP antibodies were not detected in patients with either alcohol-induced chronic pancreatitis or intraductal papillary mucinous neoplasm. *H. pylori* PBP was found to have substantial homology with ubiquitin-protein ligase E3 component n-recognin 2 (UBR2), an enzyme highly expressed in the acinar cells of the pancreas, and thus this could be another pathway through which *H. pylori* provokes molecular mimicry-induced autoimmune pancreatitis. The following year, our group (Löhr *et al*[30]) conducted a study on autoimmune pancreatitis samples using gene and protein expression profiling as well as immunoassays. Our research confirmed that acinar cells, in addition to ductal cells, are the target of immune-related inflammatory process-characterizing autoimmune pancreatitis, supporting a molecular mimicry mechanism between *H. pylori* PBP and human UBR2. All this data provides a solid theoretical basis for the hypothesis that gastric *H. pylori* infection can trigger autoimmune pancreatitis in genetically predisposed subjects. Moreover, in a series of patients with chronic pancreatitis, Dore *et al*[31] reported a reversal of elevated pancreatic enzymes after *H. pylori* eradication. However, although prevention and treatment strategies for autoimmune pancreatitis acknowledge *H. pylori* as the cause, or one of the causes, of this disease, future clinical studies that include a large number of cases will be needed in order to confirm these findings.

In conclusion, summarizing the data from available clinical studies supports a role for *H. pylori* in pancreatic carcinogenesis and autoimmune pancreatitis. Although the exact mechanisms are still unknown, molecular mimicry may play a role in the development of autoimmune pancreatitis, while pancreatic carcinoma may develop in response to *H. pylori* colonization of the antrum leading to an increase in secretin secretion and pancreatic bicarbonate output resulting in ductal epithelial cell proliferation. However, further research is needed to confirm these theoretical assumptions on the role of *H. pylori* in the development of pancreatic disease.

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TOPIC HIGHLIGHT

WJGP 5th Anniversary Special Issues (1): *Helicobacter pylori*

Use of probiotics in the fight against *Helicobacter pylori*

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Abstract

After the discovery of *Helicobacter pylori* (*H. pylori*), and the evidence of its relationship with gastric diseases, antibiotic-based therapies were developed, which efficacy was however limited by antibiotic resistance and lack of patient compliance. A vaccine would overcome these drawbacks, but currently there is not any *H. pylori* vaccine licensed. In the frame of the studies aimed at finding alternative therapies or at increasing the efficacy of the current ones and/or reducing their side effects, the investigation on the use of probiotics plays an interesting role. *In vitro* and preclinical studies have shown the feasibility of this approach. Several clinical trials indicated that administration of probiotics can reduce the side effects of *H. pylori* eradication treatment, increasing tolerability, and often increases the overall efficacy. The results of these trials vary, likely reflecting the variety of probiotics assessed and that of the eradication treatment, as well as the differences in the geographic area that imply different *H. pylori* strains distribution, host susceptibility, and therapy efficacy. In conclusion, the use of probiotics appears promising as an adjuvant for the current *H. pylori* eradication treatment, though it still requires optimization.

Key words: *Helicobacter pylori*; Treatment; Probiotics; *Lactobacilli*; *Bifidobacteria*

Core tip: *Helicobacter pylori* (*H. pylori*) is the only bacterium that has been linked to cancer to date. The efficacy of antibiotic-based eradication treatment is hampered by antibiotic resistance and side effects that may reduce patient compliance. No vaccine is currently licensed. Thus, administration of alternative compounds that may increase the efficacy of the treatment and/or reduce side effects is of particular interest. Administration of probiotics has been proposed to increase tolerability and efficacy of the *H. pylori* eradication treatment. The results of the most recent clinical trials seem to confirm these hypotheses.

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INTRODUCTION

The gastric mucosa of more than 50% of human population is estimated to be colonized by *Helicobacter pylori* (*H. pylori*), a curved or spiral-shaped, flagellated, microaerophilic, Gram-negative bacillus. *H. pylori* was isolated and cultured from human gastric biopsies only at the beginning of 1980s[1] and classified a few years later[2], although bacteria in mammalian stomach had been already observed at the end of 19th century. Prevalence of *H. pylori* infection is much higher in developing than in developed countries[3,4], most probably as a consequence of different hygiene and living conditions.

H. pylori colonization is mostly asymptomatic, but a subset of the *H. pylori*-infected population develops chronic gastritis, peptic ulcer, or gastric mucosa-associated lymphoid tissue (MALT) lymphoma[5-7]. Moreover, *H. pylori* infection increases the risk of developing gastric cancer, thus WHO has included this pathogen among the category 1 carcinogens[8-10]. Both direct bacterial action and host

response originate chronic inflammation of the stomach and the pathological outcome in the presence of *H. pylori* infection. To make inoffensive the strong host immune response, *H. pylori* activates escaping strategies and exerts on the host immune system immunomodulatory action, through various mechanisms, including the ability of eliciting T regulatory cells and of driving T helper type 1 (Th1) and Th17 response[11-14], but establishing in the majority of the cases a relatively harmless coexistence. Nevertheless, the concomitance of certain host genetic backgrounds (such as particular polymorphisms of inflammatory cytokines[15-18]), or particular susceptibility to develop gastric autoimmunity through the activation of CD4+ Th1 cells specific for *H. pylori* peptides cross-reactive with H+, K+-ATPase[19] and factors that make *H. pylori* particularly virulent (such as CagA, the product of cytotoxin-associated gene A[20,21]), can alter this equilibrium and lead to pathological outcomes including malignant lesions.

Diagnosis of *H. pylori* infection in symptomatic subjects is generally followed by the eradication therapy. The eradication causes regression of *H. pylori*-induced peptic ulcer and MALT lymphoma[10,22], and would represent a tool for reduction of gastric cancer incidence in risk populations[23]. Current standard therapies against *H. pylori* are based on the use of one proton pump inhibitor plus two or more antibiotics for one-two weeks[24], with several variants also according to the geographic area[25-27]. The efficacy of the treatments has decreased below 80%[28], mainly due to the increase of antibiotic resistance but also to side effects (such as nausea, vomiting, diarrhea, constipation, fever, headache, *etc.*[29]), which, although generally mild, may cause poor patient compliance and discontinuing of the treatment. Thus, modifications in the combination, sequence, and duration of drug administration are continuously under investigation[24,29].

Vaccination would represent a valid alternative approach to overcome the existing problems with the antibiotic therapy. A large number of preclinical efficacy studies for vaccine candidates against *H. pylori* have been published, which however were followed by a limited number of clinical trials[30]: unfortunately, the trials that included efficacy studies failed. Presently, there is not any licensed anti-*H. pylori* vaccine.

Probiotics include several microorganisms, mostly within *Lactobacillus* or *Bifidobacterium* genus, which can be grouped under the current definition living microorganisms, which, upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition[31,32]. The beneficial effects of probiotics on gastrointestinal diseases, including antibiotic-associate diarrhea, have been widely described[33-37]. Thus, due to the gastric localization of *H. pylori* colonization and its relationships with gastric diseases, it is not surprising that several studies were carried out on the effects of probiotics on *H. pylori*. Numerous *in vitro* studies, demonstrating bacterial killing or inhibition[38], were followed by preclinical and clinical studies[38-40]. These studies indicated only partial efficacy of probiotics against *H. pylori* when administered alone, but increase of efficacy and/or reduction of side effects when probiotics were administered together with the eradication treatment[39,40].

The present review is aimed at summarizing the results of the clinical trials reported in the last two years, which assessed the efficacy of probiotics administration as an adjuvant for *H. pylori* eradication treatment. The efficacy against *H. pylori* of probiotics administered alone will be not discussed.

META-ANALYSES

Three meta-analyses on probiotics supplementation of *H. pylori* eradication therapy were published in 2013. All three meta-analyses concluded, in agreement each other, that overall probiotics exerted beneficial effects on eradication treatment, with eradication rates significantly increased. Two of these meta-analyses observed significant decrease of side effects when probiotics were added to the eradication treatment, while one of them did not observe any variation. The variety of *H. pylori* eradication treatments and of the probiotics used makes impossible a direct comparison of the results of the single studies each other; nevertheless, the overall results may provide valuable information about the possible efficacy of probiotics.

The first analysis, by Wang *et al*[41] included 10 trials (9 in adults, 1 in children), corresponding to 1469 patients overall. Of these, incidence of side effects was reported in 6 trials corresponding to 978 patients. The analysis considered only parallel controlled trials, with confirmation of eradication outcome by urea breath test or rapid urea test, and comparing at least 2 branches of treatment consisting of control group (proton pump inhibitor plus 2 antibiotics with placebo or no additional intervention) and experimental groups (the same eradication regimen plus *Lactobacillus*-containing and *Bifidobacterium*-containing probiotic compound preparation). Eradication was observed in 82.63% (535 patients eradicated/708 treated) (intention-to-treat analysis, ITT) or 87.42% (535/612) (per-protocol analysis, PP) of the subjects receiving eradication therapy supplemented by probiotics, *vs* 67.85% (517/762, ITT) or 76.43 (496/649, PP) in the control group receiving eradication therapy alone. Side effects were observed in 15.37% (71/462, ITT) of the probiotics + therapy group, *vs* 31.01% (160/516, ITT) of the control group.

The second analysis, by Zheng *et al*[42] included 9 trials (6 in adults, 3 in children), corresponding to 1163 patients. Five of these trials, corresponding to 739 patients, reported the incidence of side effects. The analysis considered only randomized controlled trials that compared the efficacy of probiotic preparations, administered together with triple or sequential therapy, with that of placebo (or blank control) in *H. pylori*-positive participants. *H. pylori* positivity was assessed by ^{13}C-urea breath test, and/or histology, and/or stool antigen test. Eradication rate increased from 68.54% (414/604, ITT) of the control group to the 78.18% (437/559, ITT) of the

probiotics+therapy group (receiving a single *Lactobacillus* species or multi-strain compounds including *Lactobacilli*). Side effects did not show variations overall, being observed in 31.21% (108/346, ITT) of probiotics + therapy group, *vs* 34.86% (137/393, ITT) of the controls. Remarkably, significant differences were found when examining separately the subgroup of five trials in which a single *Lactobacillus* species was administered; in this case, significant increase of eradication rate was accompanied by decrease of side effects as compared to control group.

The third analysis, by Li *et al*[43] included 7 pediatric randomized controlled trials, corresponding to 508 patients. Five of them, corresponding to 393 subjects, reported the incidence of side effects. The studies included in this meta-analysis compared at least two treatment groups: one receiving triple regimen (proton pump inhibitor and two antibiotics) with placebo or no extra intervention, and one receiving the same triple regimen plus probiotics. Eradication was confirmed by urea breath test or stool antigen test or histology or rapid urea test. Probiotic preparations consisted of multi-strain compounds including *Lactobacillus* and *Bifidobacterium* species, and *S. thermophilus*; one study used *S. boulardii*. Eradication was observed in 78.13% (200/256, ITT) or 82.30% (200/243, PP) of the probiotics + therapy group *vs* 66.67% (168/252, ITT) or 69.42% (168/242, PP) of the controls; the probiotics + therapy resulted efficacious in reducing side effects to 21.72% (43/198) from 42.56% (83/195) observed in control group.

RECENT CLINICAL TRIALS

The trials reported in 2012-2013, in which *H. pylori* eradication treatments with or without probiotics administration were compared, are summarized in Table 1[44-53]. All were randomized clinical trials that included at least one *H. pylori* eradication treatment group and one group that received the same eradication treatment plus probiotic compounds.

Evidence of the ability of probiotics treatment to both significantly increase the efficacy of *H. pylori* treatment and decrease the side effects was provided in 3 out of 10 studies. Efficacy only in reducing side effects was observed in 3 out of the 9 studies for which the side effects description was available; in 1 out of these 9 studies, the efficacy on *H. pylori* eradication only was observed. In 2 out of 10 studies, inefficacy of probiotics was observed, both in increasing eradication and in decreasing side effects. In summary, efficacy against *H. pylori* was reported in 5 out of 10 studies, while in 6 out of 9 studies reduction of side effects was observed; overall, efficacy against *H. pylori* and/or reduction of side effects was observed in 8 out of 10 studies.

Interestingly, in 3 out of the 5 studies in which probiotics were ineffective to increase eradication rates, the eradication rates achieved with the treatment without probiotics were already relatively high (> 80%). Conversely, in the 5 studies in which inclusion of probiotics significant increased efficacy, the treatment in the absence probiotics gave relatively low eradication rates (< 70%).

In one of these studies[45], one group received probiotics plus lactoferrin, based on a previous study[54] which hypothesized that lactoferrin could contribute to increase eradication efficacy. No differences of eradication rates were observed between the group that received and the group that did not receive lactoferrin; however, eradication rates in all groups of this study were near 90%, thus possible improvements were difficult to observe. Moreover, lactoferrin did not influence the rate of side effects[45].

POSSIBLE MECHANISMS FOR THE EFFICACY OF PROBIOTICS IN REDUCING SIDE-EFFECTS AND/OR INCREASE EFFICACY OF *H. PYLORI* ERADICATION TREATMENT

Antibiotic-associated diarrhea is a frequent phenomenon[35]. Interestingly, diarrhea is the most common side-effect of *H. pylori* eradication therapy that results to decrease upon probiotics administration (Table 1). Antibiotics are known to induce diarrhea because they alter intestinal microflora, leading to a proliferation of resistant bacterial strains, and to impairment of the fermentation processes carried out by intestinal microorganisms[35]. Some authors have already demonstrated significant reduction of antibiotic-associated diarrhea, as well as of acute diarrhea, by using probiotic compounds[34,35,37]. The action of probiotics can be ascribed to their ability to stimulate mucosal immune mechanisms (*e.g.*, activation of local macrophages to increase antigen presentation, and modulation of cytokine profiles). For instance, administration of probiotics-containing yogurt to *H. pylori*-infected children was shown able to restore the normal *Bifidobacterium* spp./*E. coli* ratio, increment serum IgA, and reduce serum interleukin 6 (*IL-6*)[55]. Probiotics action may also be exerted via non-immune mechanisms through antagonism and competition with potential pathogens; in particular, probiotics are able to produce antioxidants and antimicrobial substances, alter local pH, stimulate mucin production, enhance intestinal barrier functions, modify pathogen-derived toxins, and may affect colonization by competing with pathogens for nutrients and for the binding to the host cell surface[37,56]. Finally, microbiota, through the gut-brain connection, have been suggested to be involved in the pathophysiology of mood and anxiety disorders, and possible role of probiotics in modulating abdominal pain has been proposed, based on studies in rats[36,57].

All these general actions of probiotics have been proposed to contribute to their efficacy in increasing *H. pylori* eradication and decreasing side effects when used together with eradication therapy[58,59]. A limited number of *in vitro* or non-clinical studies have been described in

Table 1 Summary of trials using probiotics with *Helicobacer pylori* eradication treatment (2012 to date)

Treatment (oral administration)	Probiotic(s) (oral administration)	Region	Eradication rates		% side effects	Probiotic(s) efficacy	Ref.
			Intention to treat	Per protocol			
Esomeprazole 20 mg, levofloxacin 500 mg, amoxicillin 1 g, all *bid*, 7 d	10^8 CFU *Lactobacillus reuteri*, during therapy + further 7 d	Italy	80% (36/45)	80% (36/45)	66.7	Significant increase of eradication rates and reduction of side effects (nausea and diarrhea)	[44]
	Control		62.2% (28/45)	62.2% (28/45)	100.0		
Esomeprazole 20 mg and amoxicillin 1 g, both *bid*, 5 d; then esomeoprazole 20 mg, clarithromycin 500 mg, tinidazole 500 mg, all *bid*, 5 d (sequential therapy)	10^9 CFU *L. Acidophilus*, 10^9 CFU *L. bulgaricus*, 5×10^8 CFU *Bifidobacterium bifidum*, 10^9 CFU *Streptococcus thermophilus*, *bid*, during therapy	Italy	89% (65/73)	92.9% (65/70)	39.7	Eradication rates unaffected; significant decrease of side effects (metallic taste, abdominal/epigastric pain, diarrhea). Addition of lactoferrin did not influence the results achieved with probiotics	[45]
	Probiotics as above + 200 mg lactoferrin		88.5% (69/78)	93.2% (69/74)	38.5		
	Control		88.2% (67/76)	94.4% (67/71)	65.8		
Omeprazole 1 mg/kg *sid*, amoxicillin 50 mg/kg *bid*, clarithromycin 15 mg/kg *bid*, 7 d	5×10^9 CFU *L. plantarum*, 2×10^9 CFU *L. reuterii*, 2×10^9 CFU *L. casei subsp. rhamnosus*, 2×10^9 CFU *B. infantis* and *B. longum*, 10^9 CFU *L. salivarius*, 10^9 CFU *L. acidophilus*, 5×10^9 CFU *S. thermophilus*, 10^9 CFU *L. sporogenes*, *sid*., during therapy	Italy	88.2% (30/34)	88.2% (30/34)	14.5	Non-significant increase of eradication rates; significant reduction of side effects (epigastric pain, nausea, vomiting, diarrhea)	[46]
	Control		76.4% (26/34)	76.4% (26/34)	61.5		
Omeprazole 20 mg, clarithromycin 500 mg, amoxicillin 1 g, all *bid*, 7 d	*L. acidophilus* 14 d after therapy	China	79.2% (61/77)	82.4% (61/74)	89.2	Significant increase of eradication rates; no influence on side effects	[47]
	3×10^7 *L. acidophilus* 14 d before therapy		79.5% (62/78)	81.6% (62/76)	85.5		
	Control		60.8% (48/79)	61.5% (48/78)	87.2		
Omeprazole 20 mg, bismuth subcitrate 240 mg, amoxicillin 1 g, clarithromycin 500 mg, all *bid*, 14 d (quadruple therapy)	*L. casei*, *L. rhamnosus*, *L. acidophilus*, *L. bulgaricus*, *B. breve*, *B. longum*, *S. thermophilus*, total viable count 10^8 CFU, *bid*, during therapy	Iran	76.6% (69/90)	82.1% (69/84)	18.8	No significant differences in efficacy and overall side effects (decrease of diarrhea but increase of abdominal pain)	[48]
	Control		81.1% (73/90)	84.8% (73/86)	16.6		
Standard triple therapy (details not disclosed)	3×10^9 CFU *B. infantis*, *bid*, during therapy	United Arab Emirates	83% (83/100)		3.0	Significant increase of eradication rates, and reduction of incidence of antibiotic-induced side effects (diarrhea, loose bowel motion)	[49]
	3×10^9 CFU *B. infantis*, *bid*, 14 d before therapy, then during therapy		90.5% (86/95)		2.1		
	Control		68.9% (73/106)		14.2		
Sequential therapy (details not disclosed), 10 d	3×10^9 CFU *B. infantis*, *bid*, during therapy		90.8% (69/76)		1.3		
Amoxicillin 50 mg/kg, furazolidone 6 mg/kg, both *bid*, 7 d, plus omeprazole 1 mg/kg *sid* 28 d	*L. casei*, *L. rhamnosus*, *L. acidophilus*, *L. bulgaricus*, *B. infantis*, *B. breve*, *S. thermophilus*, total viable count 10^9 CFU, *sid*, during therapy	Iran	90.1% (30/33)		21.2	Significant increase of eradication rates, and reduction of side effects (nausea, vomiting, diarrhea)	[50]
	Control		69.7% (23/33)		63.6		
Furazolidone 200 mg, tetracycline 500 mg, lansoprazole 30 mg, *bid*, 7 d	*L. acidophilus*, *L. rhamnosus*, *B. bifidum*, *S. faecium*, 1.25×10^9 CFU each, *sid*,during therapy and further 23 d	Brazil	81.8% (45/55)	89.8% (44/49)	59.3/44.9	Non-significant increase of eradication rates and non-significant reduction of side effects (at 7 and 30 d)	[51]
	control		76.9% (40/52)	85.1% (41/48)	71.2/60.4		
Standard triple therapy	*L. acidophilus*, *B. bifidum* during and after therapy	China	83.7% (36/43)			Increase of eradication rates	[52]
	Control		64.4% (29/45)				
Omeprazole 20 mg, amoxicillin 1 g, clarithromycin 500 mg, all *bid*, 14 d	2×10^8 CFU *L. reuteri*, *sid*, during therapy and further 14 d	Egypt	74.3% (26/35)	74.3% (26/35)	28.6%	Non-significant increase of eradication rates; significant decrease of side effects (taste disorders, diarrhea)	[53]
	Control		65.7% (23/35)	65.7% (23/35)	68.6%		

CFU: Colony forming unit; Control: Group that received the eradication treatment without probiotics or with placebo; the term "significant" was used when $P < 0.05$ was reported in the corresponding paper.

the literature that can help to understand possible direct and specific activity of probiotics against *H. pylori*. The most recent studies are described below.

H. pylori urease catalyzes the conversion of urea to carbon dioxide and ammonia; ammonia in turn forms ammonium hydroxide, which neutralizes the local acidity in favor of *H. pylori* survival. Some studies reported the ability of *Lactobacillus casei* (*L. casei*) to inhibit *H. pylori* ure-

ase[60,61]; the specific effect on urease was suggested by the fact that such inhibition was observed under experimental conditions that did not influence the bacterial growth. This activity may be due to the activity of lactic acid[60]. More in general, anti *H. pylori* activity exerted by lactic acid bacteria has been proposed to be due to organic acids produced by these bacteria[56,60,62].

Recently, some *Lactobacillus* strains (*L. gasseri* Chen and *L. plantarum*) have been reported to be able to inhibit *H. pylori* adherence to gastric epithelial cells[63]. Similar results were described for some *Lactobacillus* strains (including *L. acidophilus*, *L. johnsonii*, and *L. salivarius* subsp. *salicinius*) that were able to reduce *H. pylori* adhesion to the human gastric adenocarcinoma cell line AGS, and also its intracellular growth; generally, this activity was more evident using culture supernatants of *Lactobacilli* rather than using bacterial cells[64]. *L. salivarius* was also able to counteract the increase of IL-8 production induced by *H. pylori* in AGS cells[64]. Administration of *L. johnsonii* or *L. salivarius* to rats infected by *H. pylori* revealed a reduction of bacterial load, of local IL-8 production, and of gastric inflammation[64]. Moreover, *L. johnsonii* La1 culture supernatant was found able to reduce *H. pylori* motility and its adherence to the human gastric epithelial cell line MKN74, providing a possible explanation of the ability of *L. johnsonii* La1 to reduce gastric colonization in *H. pylori*-infected Mongolian gerbils[65]. In the same animal model, long-term administration of yogurt supplemented with probiotics (*L. acidophilus, L. bulgaricus, B. lactis, S. thermophilus)* was found to reduce *H. pylori* colonization, TNF-α expression, gastric inflammation and intestinal metaplasia as compared with infected controls not receiving probiotics[66].

Probiotics may also interfere with the activation of specific host pathways by *H. pylori*. *L. acidophilus* produces conjugated linoleic acids (CLA) that have been shown by some studies able to interfere with inflammatory outcomes of *H. pylori* infection[67]. This interference targets nuclear factor-κB pathway[67,68], which is known to be induced by *H. pylori*. Consistently with these observations, CLA from *L. acidophilus* or *L. plantarum* was also shown to suppress the *H. pylori*-induced IL-8 and TNF-α expression by the AGS cell line[69].

Further studies showed that *L. acidophilus* can also interfere with the Smad7 activation, also in this case resulting in reduced inflammatory events[68]. Conditioned media from *L. salivarius*, *L. rhamnosus*, and *L. plantarum* were found to suppress the *H. pylori*-induced Il-8 expression and NFκB activation in AGS cells, without inhibiting *H. pylori* growth; *L. plantarum* was also able to suppress the activation of *c-jun* (which is one of the proto-oncogenes activated by *H. pylori* CagA[70]) *in vitro*, and to attenuate gastric inflammation in a rat model of *H. pylori* infection[71].

CONCLUSION

Probiotics are generally considered safe to administer to humans, and several strains have already received indication for use in specific disorders[37]. Probiotics treatment as an adjuvant of eradication treatment showed in the recent trials efficacy against *H. pylori* and/or decreased side effects of the treatment in most of the studies - but not in all. This confirms the previously reported results. It must be remarked that the efficacy of probiotics treatment in increasing eradication can be evaluated only when eradication rates in the controls that did not receive probiotics are low enough; on the other hand, the efficacy in reducing side effects can be observed when side effects are present, *i.e.,* in almost all studies. To date, it does not appear clear whether probiotics may be more effective in particular subgroups, and if predictive factors for treatment success can be identified. The meta-analysis by Zheng *et al*[42] suggested that using single *Lactobacillus* species could achieve better results than administering multi-strain compounds; however, this was not highlighted by other meta-analyses, and remains a point to be further clarified. Possible influence of age, lifestyle (dietary habit in particular), grade of infection, type of gastroduodenal symptoms, and other similar factors could be analyzed in wider meta-analyses, as this information is provided at least in part in the reports of the clinical trials. Conversely, the possible influence on probiotics efficacy of essential factors such as for instance the *H. pylori* infecting strain, the host genetic background, and the host microbiome, could be assessed only by studies specifically designed to investigate the relevance of these factors.

It is known that *H. pylori* isolates are different according to the geographic areas, and that the susceptibility to *H. pylori* infection and the outcome of the infection vary according to both *H. pylori* and/or host genetic background, that may result in combinations much more harmful than others[17,18,21], and may also influence the eradication rates achievable. Thus, it is not unexpected that some studies, in disagreement with others, did not find beneficial effects of probiotics adjunctive treatment: having more information of *H. pylori* isolates and on genetic background of the hosts would strongly help to understand the reasons of success or of failure of probiotics.

Possible specific activity of probiotics against defined *H. pylori* factors is still largely to be understood. Indeed, the decrease of inflammatory cytokines, restoration of IL-10, suppression of NFκB activation, *etc.*, in the majority of the cases may be indirect effects, *i.e.,* related to the ability of probiotics to reduce *H. pylori* adhesion (*in vitro* studies) or colonization (*in vivo* studies). To date, the only proposed possible specific *H. pylori* target for probiotics has been urease[60,61]. It would be interesting to experimentally assess the possible interference of probiotics or probiotics factors with the *in vitro* and/or *in vivo* activities of other well-characterized *H. pylori* factors such as for instance CagA and VacA, besides urease. However, it must be said that probiotics may have low chance of entering in direct and massive contact with *H. pylori* as the latter resides under the mucus layer of the gastric mucosa, in large part adherent to the epithelial cells, where probiotics are unlikely to arrive in significant amount. In fact, the optimal conditions for probiotics colonization are present

in the large intestine, where the highest concentration of probiotics is found, while scarce concentration of probiotics is usually found in the stomach[72]. Thus, at least for therapeutic use, it seems more likely that probiotics exert indirect and non-specific rather than direct and specific anti-*H. pylori* activity.

In conclusion, administration of safe probiotics as an adjuvant for the current *H. pylori* eradication treatment appears promising, though it still requires optimization; even in the cases in which the treatments achieve high eradication rates, probiotics may reduce side effects. Further investigation on the mechanisms behind the direct and indirect effects of probiotics on *H. pylori* could help not only to better refine the type of treatment, but also may contribute to better understand some aspects of *H. pylori* pathogenesis.

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TOPIC HIGHLIGHT

WJGP 5th Anniversary Special Issues (1): *Helicobacter pylori*

Treatment of *Helicobacter pylori* infection: Past, present and future

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Abstract

Helicobacter pylori (*H. pylori*) is a major human pathogen associated with significant morbidity and mortality. However, after decades of efforts, treatment of *H. pylori* remains a challenge for physicians, as there is no universally effective regimen. Due to the rising prevalence of antimicrobial resistance, mainly to clarithromycin, efficacy of standard triple therapies has declined to unacceptably low levels in most parts of the world. Novel regimens, specifically experimented to improve the therapeutic outcome against antibiotic-resistant *H. pylori* strains, are now recommended as first-line empirical treatment options providing high efficacy (reportedly > 90% in intention to treat analysis) even in high clarithromycin resistance settings. These include the bismuth quadruple, concomitant, sequential and hybrid therapies. Due to the rapid development of quinolone resistance, levofloxacin-based regimens should be reserved as second-line/rescue options. Adjunct use of probiotics has been proposed in order to boost eradication rates and decrease occurrence of treatment-related side effects. Molecular testing methods are currently available for the characterization of *H. pylori* therapeutic susceptibility, including genotypic detection of macrolide resistance and evaluation of the cytochrome P450 2C19 status known to affect the metabolism of proton pump inhibitors. In the future, use of these techniques may allow for culture-free, non-invasive tailoring of therapy for *H. pylori* infection.

Key words: *Helicobacter pylori*; Antibiotic resistance; Bismuth-quadruple; Concomitant; Sequential; Probiotics

Core tip: Worldwide increase in prevalence of macrolide resistance has accounted for the failure of standard therapies for the treatment of *Helicobacter pylori* (*H. pylori*) infection. Bismuth quadruple, concomitant, sequential and hybrid therapies are now recommended as first-line empirical treatments providing improved efficacy in high clarithromycin resistance settings. As quinolone resistance is rapidly increasing, levofloxacin should be preferentially used in second-line/rescue therapies. There is increasing evidence that adjunct probiotic supplementation improves the therapeutic outcome and tolerability. Genotypic characterization of *H. pylori* susceptibility to therapy may allow for a tailored therapeutic approach in the future.

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INTRODUCTION

Treatment of *Helicobacter pylori* (*H. pylori*) infection is paramount for the management of prevalent gastrointestinal

Table 1 Factors reported to negatively affect the outcome of therapies for *Helicobacter pylori* infection

Pathogen-related	Host-related
Development of resistance to antibiotics	Non-compliance to treatment
High bacterial load in the stomach	Non-ulcer dyspepsia
Protective effect of the gastric mucus layer	Smoking
Intracellular location of many bacteria	CYP2C19 status (rapid metabolizer)
CagA negative	
Presence of dormant coccoid forms (not susceptible to antibiotics)	
Heteroresistant status (co-existence of strains susceptible and resistant to the same antibiotic)	

diseases, including peptic ulcer disease, gastric cancer and functional dyspepsia[1-3]. Moreover, extra-digestive disorders are now included as indications for eradication of *H. pylori*: idiopathic thrombocytopenic purpura, vitamin B12 deficiency and unexplained iron deficiency anemia[4]. Contrarily to other bacterial infections, for which susceptibility testing is commonly performed to guide treatment, culture of *H. pylori* is not widely available and requires performing endoscopy which is not well-tolerated by all patients and has a series of limitations, including the fact that *in vitro* susceptibility does not always guarantee *in vivo* eradication[5]. Hence, regimens for *H. pylori* have been routinely prescribed empirically, provided they have been previously tested and sufficiently tailored with regard to various parameters (*i.e.*; treatment dose, duration, dosing intervals *etc.*) to optimize cure rates and minimize side effects. However, the optimal treatment to eradicate *H. pylori* remains to be established, as no regimen is effective universally. Worldwide increase in resistance to key antibiotics, mainly clarithromycin (CAM), but also metronidazole (MNZ) and levofloxacin, is the main determinant of failure in the treatment of *H. pylori* infection[6,7]. In a recent systematic review, the global incidence of CAM resistance has been reported to be 17.2% ranging from 11.1% in Europe to 29.3% in America, whereas, in the same analysis, continental rates of resistance to MNZ were 17% and 44.1% respectively[8]. Antibiotic consumption for infections other than *H. pylori* is accounting for the wide increase in *H. pylori* antibiotic resistance rates[9,10]. Indeed, different national policies for antibiotic use are largely reflecting geographical distribution of *H. pylori* resistance: CAM resistance has been reported to be significantly higher in Southern European countries (reaching 49% in some areas of Spain) as compared to Northern Europe (*e.g.,* only 1% in the Netherlands) where policies for antibiotic use are more stringent[9]. Additionally to the development of antibiotic resistance, a series of both host and pathogen related factors may negatively impact on the performance of regimens to eradicate *H. pylori* (Table 1)[11,12].

Despite decades of efforts, treatment of *H. pylori* infection remains a challenging issue for both researchers and practicing physicians. In the present article we aim to provide a comprehensive overview of perspectives on the past, present and future of *H. pylori* eradication.

CLARITHROMYCIN-BASED TRIPLE THERAPIES: A DECLINING CLINICAL STANDARD

Historically, the first truly effective therapy for *H. pylori* infection, comprising of bismuth, tetracycline and MNZ, was proposed in 1990[13]. A few years later, use of CAM in a triple therapy, proposed by Bazzoli *et al*[14], was the start of CAM-based triple regimens, thereafter representing the gold standard in the treatment of *H. pylori*. In studies conducted during the 90's, standard triple therapies (STT) comprising of a proton pump inhibitor (PPI) *bid*, CAM 500 mg *bid* and amoxicillin 1000 mg *bid* or MNZ 500 mg (or 400 mg in England), all given for 7-14 d, provided consistently good results yielding > 80% eradication success and even > 90% was feasible[15,16]. Due to this high efficacy and relative simplicity, optimal safety profile, and large pharmaceutical company commitment, these regimens have been widely accepted in national expert panels and consensus recommendations worldwide as standard of care treatments for first-line eradication of *H. pylori*[17-20]. However, rising prevalence of CAM resistance has accounted for a significant decline in the efficacy of standard regimens. This decreasing efficacy was already evident in the meta-analyses published by the early 2000's, prompting significant changes in the paradigm of treating the infection. These included the introduction of the concept of cumulative treatment efficacy (requiring the patient to comply with more treatment courses; thus, more side effects and spreading of secondary antibiotic resistance), and later the introduction of a local threshold (15%-20%) of CAM resistance at which CAM should not be used empirically[17,18]. The decreased efficacy of standard treatments against CAM-resistant strains has been well-documented on a meta-analytic basis: In a meta-analysis by Fischbach and Evans, the success of triple therapy was decreased by 66.2% (95%CI: 58.2%-74.2%) when strains of *H. pylori* were resistant *vs* susceptible to CAM[7]. Congruently, a more recent analysis by Venerito *et al*[21], revealed similar results: including antimicrobial susceptibility data from 4 randomized clinical trials (RCTs), standard triple therapies successfully eradicated 88% of CAM-sensitive but only 14% of CAM-resistant *H. pylori* strains (risk difference = 0.75, 95%CI: 0.63-0.87). If MNZ is used, presence of MNZ resistance may also affect the therapeutic outcome[22], although it is generally considered less important clinically. This is due to the fact that MNZ resistance may be largely overcome by increasing dose and prolonging treatment duration[23]. Lastly, *H. pylori* resistance to amoxicillin is exceptional and generally is not relevant clinically. In the light of increasing data confirming suboptimal performance (< 70%) in most

Table 2 Current regimens to treat *Helicobacter pylori* infection

Treatment	Regimen
Bismuth-containing quadruple therapy	A PPI (standard dose, *bid*), bismuth (standard dose, *qid*) tetracycline (500 mg, *qid*) and metronidazole (500 mg, *qid*) for 10-14 d
Sequential therapy	A 5-d dual therapy with a PPI (standard dose, *bid*) and amoxicillin (1 g, *bid*) followed by a 5-d triple therapy with a PPI (standard dose, *bid*), clarithromycin (500 mg, *bid*) and metronidazole (500 mg, *bid*)
Concomitant therapy	A PPI (standard dose, *bid*), clarithromycin (500 mg, *bid*), amoxicillin (1 g, *bid*) and metronidazole (500 mg, *bid*) for 7-10 d
Hybrid therapy	A 7-d dual therapy with a PPI (standard dose, *bid*) and amoxicillin (1 g, *bid*) followed by a 7-d quadruple therapy with a PPI (standard dose, *bid*), amoxicillin (1 g, *bid*), clarithromycin (500 mg, *bid*) and metronidazole (500 mg, *bid*)
Levofloxacin-based triple therapy	A PPI (standard dose, *bid*), levofloxacin (500 mg, *bid*) and amoxicillin (1 g, *bid*) for 10 d

PPI: Proton pump inhibitor.

European countries, the recent Maastricht IV/ Florence consensus report has definitively displaced standard regimens as the empirical gold standard to eradicate *H. pylori*[4]. Instead, use of legacy triple regimens should take into account the local resistance pattern (thus, used only in areas in which CAM resistance is < 20%) or rely on susceptibility testing provided that pre-treatment culture is available (*i.e.*, used as tailored treatments).

CURRENT THERAPIES FOR *H. PYLORI* INFECTION

Novel regimens, specifically experimented to improve the therapeutic outcome against antibiotic-resistant *H. pylori* strains, are now recommended as first-line empirical treatment options providing improved efficacy (reportedly > 90% in intention to treat analysis) in high CAM resistance settings. These regimens are summarized in Table 2.

BISMUTH QUADRUPLE THERAPY

Bismuth quadruple therapy (BQT) currently represents a preferred first-line treatments option for areas with a high (≥ 20%) incidence of CAM resistance but also a valuable second-line treatment option when a CAM-based regimen has previously failed. It works independently to CAM achieving > 90% eradication in the presence of CAM resistance, whereas implementation of a high MNZ dose (1500-1600 mg/d) and prolonged (10-14 d) treatment duration allow for minimizing the impact on MNZ resistance, providing eradication rates > 85% even in regions with a high resistance to this drug[24]. A patient-friendly monocapsule (containing bismuth, MNZ and tetracycline) is available (Pylera®, Aptalis, Mont St Hilaire, QC, Canada) providing intention-to-treat eradication rates of 86% and 80% in two large RCTs conducted in North America and Europe respectively[25-27]. Contrarily, the ITT eradication rate with BQT was only 77.8% in a recent meta-analysis (*vs* 77% for STT), questioning both the efficacy as well as the superiority of the BQT over STT[28]. However, a substantial grade of study heterogeneity, especially with respect to MNZ dosing, should be acknowledged. The second-line efficacy of BQT has been also confirmed on a meta-analytic basis (30 studies) showing an average 77% second-line efficacy (ITT) after failure of STT[29]. Third-line efficacy of BQT after two previous eradication failures with CAM- and levofloxacin-containing triple therapies was 65% (ITT) in a multi-center study from Spain[30]. Non-availability of bismuth salts or tetracycline in some countries as well as the potential toxicity of bismuth are the main limitations. However, including 4763 patients no differences with respect to tolerability were shown between non-bismuth and bismuth-containing groups except from dark stools being more common in the later[31].

SEQUENTIAL THERAPY

Sequential therapy uses the same antibiotics contained in STT but administered sequentially. It has been postulated that the initial course of amoxicillin disrupts the bacterial cell wall preventing the development of efflux channels transferring CAM out of the bacteria[32]. Although in the initial RCTs[33] (most of them conducted in Italy) and earlier meta-analyses sequential therapy was clearly superior to STT [ITT eradication 91.7% (95%CI: 90%-93%) *vs* 76.7% (95%CI: 75%-79%) for STT][34], more recent data from South America, Iran and South Korea revealed lower eradication rates (< 80%)[35-37]. Despite this sequential therapy seems to be fairly effective against CAM mono-resistant strains, being able to eradicate 72.8% of them, its efficacy against dual resistant (CAM and MNZ) strains was only 37% (range: 16.2% to 60.7%) when 8 studies with antibiotic susceptibility data were evaluated[38]. Critically, sequential therapy was not superior to either a 14-d triple therapy (RR = 1, 95%CI: 0.94-1.06) or a bismuth-based therapy (RR = 0.99, 95%CI: 0.94-1.05) in an extensive evaluation of 46 RCTs[38].

NON-BISMUTH QUADRUPLE (CONCOMITANT) THERAPY

A non-bismuth quadruple "concomitant" therapy is another valid first-line treatment option for areas with a high incidence of CAM resistance[39,40]. In 19 studies (2070 patients) the overall eradication rate with concomitant therapy was 88% (95%CI: 85%-91%) and 91% when 3 outlying studies with inherently short treatment duration (3-5 d) were excluded[41]. Indeed, treatment duration of

at least 7 d has been shown to be necessary for the success of concomitant therapy[42], whereas extra-prolonging treatment to 14 d combined with a high PPI dose (omeprazole 40 mg × 2) may further boost cure rates to > 95%, as revealed by a non-inferiority multi-center trial[43]. An increased efficacy against dual resistant *H. pylori* strains has been proposed as the main strength of the concomitant over the sequential therapy[44], though the two regimens have performed equally when compared using 338 patients in a high antibiotic resistance country (Spain)[45]. Indeed, by evaluating 106 patients with pre-treatment susceptibility testing, the concomitant therapy eradicated only 55% of dual-resistant strains *vs* 100% and 91% with CAM and MNZ resistance respectively[46]. Thus, both regimens seem to be prone to the deleterious impact of dual resistance, performing comparably (with about 81% of efficacy each) by pooling data of 6 comparative RCTs[38].

HYBRID THERAPY

A two-step dual-concomitant (hybrid) regimen, proposed by Hsu *et al*[47], is another valuable treatment option competing with both the sequential and concomitant treatments. By evaluating data from 2 RCTs, hybrid therapy performed marginally, though not significantly, better as compared to sequential therapy (86.6% *vs* 81%)[38], and comparably to concomitant therapy in a comparative study in which, interestingly, fewer adverse events occurred in the group treated with the hybrid regimen[43]. Further data is warranted to allow for definitive conclusions on the efficacy and tolerability of hybrid therapy.

LEVOFLOXACIN-BASED THERAPIES

To overcome increasing CAM resistance, levofloxacin, a broad spectrum quinolone, has been used as a substitute of CAM in either triple or sequential regimens achieving > 90% cure rates, and even > 95% is feasible provided that the local resistance to levofloxacin is low (< 10%)[48,49]. However, levofloxacin also encounters clinically significant problems of antibiotic resistance, as resistance to quinolones currently exceeds 40% in America, 20% in Europe and 10% in Asia[8]. Due to the rapid development of secondary quinolone resistance, first-line use of levofloxacin is generally discouraged, and the drug is reserved for use in second-line/rescue regimens after failure of a CAM- and/or a MNZ-based regimen[50]. The good (cure rates 81%-87%) second-line efficacy of a levofloxacin triple therapy (LTT) has been confirmed in two meta-analyses published in 2006, both showing better results with LTT in comparison with second-line BQT[51,52]. Congruently, second-line efficacy of LTT was 88.7% in a more recent meta-analysis including RCTs up to October 2010[53]. Critically, use of LTT after failure of either a sequential or concomitant regimen has been reported to provide up to 97.8% of cumulative therapeutic efficacy[54]. Use of other quinolone agents, such as Moxifloxacin and Sitafloxacin, has shown promising results[55,56], though there is no evidence to support any therapeutic advantage over levofloxacin.

FUTURE PERSPECTIVES

Adjunct probiotics

Albeit different attempts have been made to restore the efficacy of standard treatments, such as increasing the PPI dose or prolonging treatment duration, none has been proved at a level to overcome today's antimicrobial resistance. An approach which has attracted growing interest is using probiotics in conjunction with regimens to eradicate *H. pylori*[57]. The expected benefit is twofold: boosting eradication and improving tolerability by preventing occurrence of treatment-related side effects. The pathogenic basis of a possible beneficial effect of probiotics on *H. pylori* eradication remains to be clarified, though some hypothesis have been put forward including strength of the mucosal barrier, competition for adhesion and immunomodulatory mechanisms[58]. Different trials used probiotics adjunctively to either standard or novel regimens in recent years providing contradictory results[59-62]. Although different single- or multi-strain compounds have been evaluated, there is currently evidence to support use of *Saccharomyces boulardi* (OR = 1.13; 95%CI: 1.05-1.21) or *Lactobacillus spp.* (OR = 1.78; 95%CI: 1.2-2.6) supplementation adjunctively to standard triple therapy[63,64]. In the most recent analysis assessing the effect of Lactobacillus-containing and Bifidobacterium-containing supplementation, the pooled odds ratio (ITT) with probiotic supplementation was 2.066 (95%CI: 1.398-3.055) for eradication and 0.305 (95%CI: 0.117-0.793) for the incidence of total side effects[65]. Interestingly, with respect to the prevention of side-effects, use of probiotics may be relevant only in a subset of patients, in particularly those with recurrent infection or history of gastrointestinal antibiotic-related side effects[57]. Further data is awaited to clarify the role, standardize regimens and assess the cost-effectiveness of probiotics in the treatment of *H. pylori* infection.

Culture-free, non-invasive determination of H. pylori antibiotic susceptibility

Critically, even the novel treatments discussed above are to some (although to a lesser as compared to legacy therapies) degree prone to the impact of antibiotic resistance; eradication rates > 95% are infrequent, and even > 90% are disputed in some studies[35,66,67]. Furthermore, it is possible that the success of empirical treatments will further decline in the future as resistance to key antibiotics is constantly growing worldwide. In order to maintain high therapeutic efficacy, tailored treatment of *H. pylori* infection based on pre-treatment susceptibility testing appears as the ideal approach. This will prevent exposing the patient to repeated empirical treatments which increase the risk for side effects and promote development of secondary resistance. However, as

mentioned, current means of performing endoscopy and *H. pylori* culture are invasive, do not 100% reflect in vivo susceptibility, and are time-consuming as culture requires 3-10 d and susceptibility testing (*e.g.*, by Etest, AB bioMerieux, Solna, Sweden) will require additional 3-4 d. These limitations preclude systematical performance of *H. pylori* culture, which is currently recommended only for cases with at least two empirical treatment failures. A class-wide resistance to macrolides is the result of point mutations in three adjacent nucleotide positions (*A2143G, A2142G and A2142C*) in the peptidyl transferase loop of the *23SrRNA* gene[68,69]. These three point mutations account for 90% of cases of primary CAM resistance in Western countries. In recent years, molecular testing methods have been developed for these mutations including a standard polymerase chain reaction (PCR) and other PCR-based methods including PCR-restriction fragment length polymorphism, PCR-DNA enzyme immunoassay, PCR oligonucleotide ligation assay and PCR-line probe assay, as well as Real-time PCR assay which represents a powerful advancement of the basic PCR[70-72]. These methods may offer rapid and highly accurate results in the genotypic detection of CAM resistance, including detection of the heteroresistant status (*i.e.*, co-existence of strains susceptible and resistant to the same antibiotic) known to account for a significant number of treatment failures[73,74]. These techniques can be directly applied on gastric biopsy specimens or used in association with minimally-invasive techniques (*e.g.,* oro-gastric brushing or gastric wash) or non-invasively using stool specimens[75-77]. Importantly, genotypic detection of CAM resistance is also possible with Fluorescence In-Situ Hybridization, which can be also applied on paraffin-embedded specimens[78,79]. Detection of levofloxacin resistance based on the detection of *gyrA* mutations is also available[80]. Two Asian studies have provided data on the potential utility of a tailored therapeutic approach based on the molecular detection of *H. pylori* resistance to CAM. Tailored treatment using a simple PPI/MNZ regimen successfully eradicated the pathogen in 94.3% *vs* 71.4% using empirical standard treatment[77]. In a larger study (218 patients), CAM was replaced by MNZ in the triple regimen if a CAM-resistant strain was detected. Eradication rates were 91.2% in the tailored group *vs* 79.1% and 75.9% by using empirical MNZ- and CAM-based triple therapies (n = 308 in each control group) respectively ($P < 0.001$)[81].

Pharmacogenomics

Genetic variability in the activity of the cytochrome P450 (CYP) 2C19 (CYP2C19) is known to influence the plasma levels of PPIs, and thus treatment of *H. pylori* infection[82,83]. Three distinct genotypes are recognized: rapid, intermediate and poor metabolizers. Preliminary data on the potential use of pharmacogenomics has been provided by a RCT with 300 *H. pylori*-positive patients randomized to either a 1 wk standard regimen or to personalized therapy based on both CYP2C19 and CAM susceptibility status assessed by genetic testing[84]. The ITT eradication rates were significantly higher in the tailored group (96% *vs* 70%) without an increase of the final per-patient cost for successful eradication. In the future, both practical and logistic issues should be addressed before a molecular-based approach can be widely adopted as a genuine basis for the individualization of *H. pylori* eradication therapies.

CONCLUSION

For more than a decade, triple regimens have been the standard of care therapies for *H. pylori* infection. However, in more recent years, rising prevalence of macrolide resistance has accounted for a significant decline in the performance of these regimens, resulting in the necessity of more treatment courses in order to eradicate the pathogen. In order to maintain high therapeutic efficacy, regimens with an improved performance against antibiotic-resistant *H. pylori* strains are now recommended as preferred first-line treatments. The concomitant and sequential regimens are currently the best validated first-line therapeutic options. Hybrid therapy is another effective CAM-based alternative and a relevant competitor to both these treatments. BQT is also a valid treatment for high CAM resistance settings, but also an effective second-line regimen when a CAM-based regimen fails. Due to the rapid development of quinolone resistance, levofloxacin-based regimens should be currently reserved as second-or-more-line treatment options. While efforts to improve empirical treatments continue, the fields of genotypic detection of *H. pylori* antimicrobial susceptibility and pharmacogenomics offer a fascinating new perspective. This is to guarantee 100% therapeutic efficacy: fast, culture-free and non-invasive.

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TOPIC HIGHLIGHT

WJGP 5th Anniversary Special Issues (1): *Helicobacter pylori*

Helicobacter pylori and neurological diseases: Married by the laws of inflammation

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Abstract

The purpose of this paper is to review current information about the role of inflammation caused by *Helicobacter pylori* (*H. pylori*) infection in neurological diseases such as Parkinson's disease, Alzheimer's disease, Guillain-Barré syndrome, multiple sclerosis, and other inflammatory diseases including ischemic stroke. Infection with *H. pylori* usually persists throughout life, resulting in a chronic inflammatory response with local secretion of numerous inflammatory mediators including chemokines [interleukin (IL)-8, macrophage chemotactic protein (MCP)-1, growth-regulated oncogene (GRO)-α] and cytokines [IL-1β, tumor necrosis factor (TNF)-α, IL-6, IL-12, interferon (IFN)-γ], which can pass into the circulation and have a systemic effect. The persistence of detectable systemic and local concentrations of inflammatory mediators is likely to alter the outcome of neurological diseases. These pro-inflammatory factors can induce brain inflammation and the death of neurons and could eventually be associated to Parkinson's disease and also may be involved in the development of Alzheimer's disease. However, most neurological diseases are the result of a combination of multiple factors, but the systemic inflammatory response is a common component and determinant in the onset, evolution, and outcome of diseases. However, more studies are needed to allow understanding of the effects and mechanisms by which the inflammatory response generated by *H. pylori* infection affects neurological diseases.

Key words: Gastrointestinal diseases; *Helicobacter pylori*; Immune disease; Mediators of inflammation; Neurodegenerative diseases

Core tip: Neurological diseases such as Parkinson, Alzheimer, Guillain-Barré syndrome, multiple sclerosis, and ischemic stroke are the result of a combination of multiple factors, but the chronic and systemic inflammatory response to *Helicobacter pylori* could be a common component and determinant in the onset, evolution, and outcome of these diseases.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a Gram-negative bacterium that chronically infects more than 50% of the human population[1]. It is well known that infection with the bacterium increases the risk of gastric diseases including peptic ulcers and gastric cancer[2,3]. *H. pylori* is able to infect and live persistently in the human stomach and elicits

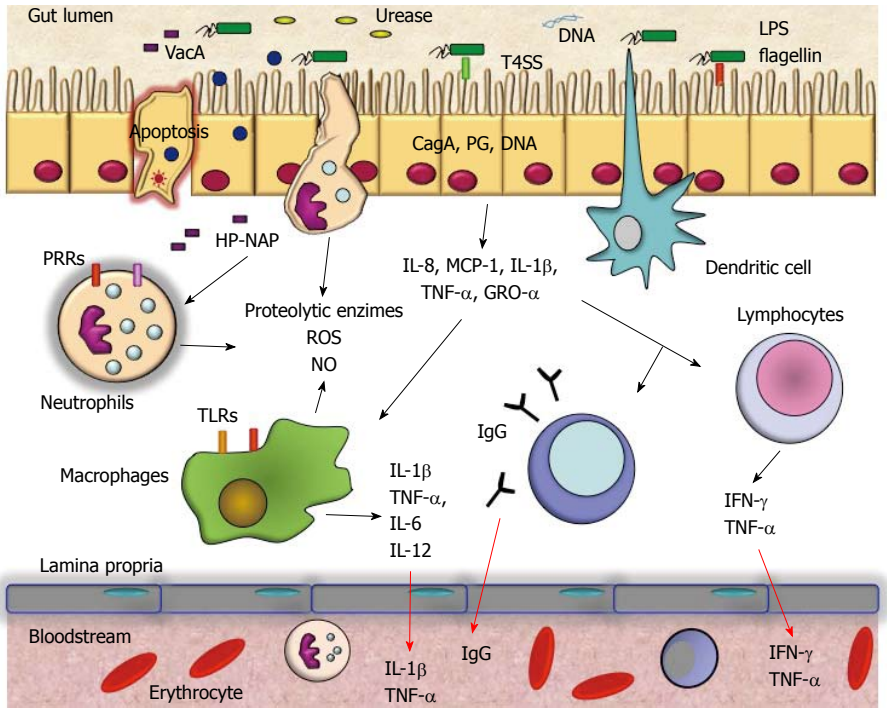

Figure 1 The inflammatory response in *Helicobacter pylori* infection. Immune cells are recruited to the lamina propria of the gastric epithelium by chemokines and cytokines (IL-8, MCP-1, GRO-α, IL-1β, TNF-α) produced by epithelial cells or directly by bacterial products including *H. pylori* neutrophil-activating protein, VacA, and urease. At the site of infection, the immune cells are activated and exert their effector functions, including the production of cytokines (IL-1β, TNF-α, IL-6, IL-12, IFN-γ, chemokines (IL-8, MCP-1), proteolytic enzymes, oxide nitric (NO) and reactive oxygen species (ROS). PG: Peptidoglycan; T4SS: Type IV secretion system; IL: Interleukin; TNF: Tumor necrosis factor; MCP: Macrophage chemotactic protein; GRO: Growth-regulated oncogene.

severe acute and chronic inflammatory responses, which may be of variable magnitude depending on the host' s genetic makeup and lifestyle[4]. Differing inflammatory responses among hosts may help to explain the different outcomes of infection with *H. pylori*[5]. *H. pylori* induces infiltration to the gastric mucosa of neutrophils, macrophages, dendritic cells, T and B cells, and stimulates secretion of interleukin (IL)-8, tumor necrosis factor (TNF)-α, IL-6, IL-1β, IL-12, IL-10, and interferon (IFN)-γ[5]. This persistent response causes significant changes in the physiology of the stomach by direct damage to the cells or by regulating cell proliferation and apoptosis (Figure 1). Neutrophils and macrophages release reactive oxygen and nitrogen species, which may induce irreversible changes in the gene expression of cells of the gastric mucosa. The levels of these chemical species decrease when *H. pylori* infection is eradicated[6]. The secretion of inflammatory mediators is likely to have serious biological implications at the local and systemic level. For example, IL-8 is a chemokine induced early by *H. pylori* infection. It is a chemoattractant of neutrophils and mediates responses to bacterial infection and to autoimmune disease[7]. IL-6 activates target genes involved in cellular differentiation, survival, apoptosis, and proliferation[8]. IL-6 may perpetuate inflammation by inducing antiapoptotic signals mediated by signal transducer and activator of transcription 3. IL-1β signaling is absolutely necessary for the efficient control of *H. pylori* infection. IL-1R(-/-) mice failed to developed protective immunity against Helicobacter-associated gastritis and gastric preneoplasia as a result of their inability to generate Helicobacter-specific T helper (Th)1 and Th17 responses[9].

IL-10 is an anti-inflammatory cytokine that inhibits the production of proinflammatory cytokines by inhibition of Th1 lymphocytes and stimulation of B cells and Th2 lymphocytes, and consequently it downregulates the inflammatory response[10]. This cytokine is very important for the maintenance of a balanced response in gastric inflammation. In contrast, IFN-γ mediates responses to bacterial infection and autoimmune disease. It is upregulated in the gastric mucosa by chronic Helicobacter infection[11]. This cytokine is important in the production of gastric acid and its levels are correlated with the damage found in gastritis.

H. pylori infection has been associated with the development and progression of neurological diseases, principally by inducing systemic inflammation, molecular mimicry, and interference with the absorption of drugs. In this review, we summarize the most important research on this issue.

PARKINSON'S DISEASE

Parkinson's disease is the second most common neurodegenerative disease worldwide. It is characterized by the accumulation of cytoplasmic proteins, including α-synuclein, which leads to the progressive loss of dopaminergic neurons. The loss of dopaminergic neurons causes the resting tremors, rigidity and bradykinesia that are characteristic symptoms of the disease[12]. *H. pylori* infection may

increase the risk of Parkinson's disease[13]. The administration of L-3,4-dihydroxyphenylalanine (L-dopa), a precursor of dopamine, is used as a treatment for Parkinson's disease. *H. pylori* infection may affect the bioavailability of L-dopa by disrupting the duodenal mucosa, which is the site of primary absorption of L-dopa[14,15]. Recent studies suggested that *H. pylori* eradication in patients with Parkinson's disease might improve the bioavailability of L-dopa and reduce motor fluctuations[15-18].

Parkinsonism is a neurological syndrome that shares symptoms found in Parkinson's disease. It has been suggested that a peripheral immune response characterized by the presence of proinflammatory cytokines such as IL-8, IL-1β, and TNF-α in the bloodstream induces a disruption of the blood-brain barrier and promotes microglia-mediated inflammation and neurotoxicity[19-21]. Several studies have established that that proinflammatory factors associated with chronic gastrointestinal disease can induce brain inflammation and the death of dopaminergic neurons and could eventually be responsible for parkinsonism[22-24]. Dobbs *et al*[25] proposed that *H. pylori* infection predisposes to autoimmunity that results in neuronal damage leading to eventual parkinsonism. This was based on the observation of an age-associated increase in the levels of antibodies against *H. pylori* in Parkinson's patients, but this association is not clear, and other investigations are required to clarify it.

ALZHEIMER'S DISEASE

Alzheimer's disease is a progressive neurodegenerative disease characterized by both synaptic loss and neuronal death as a result of extracellular and intracellular accumulation of β-amyloid deposits and neurofibrillary tangles in brain regions important for memory and cognitive processes[26]. The inflammatory response plays an important role in the pathophysiology of Alzheimer's disease. Higher levels of *H. pylori*-specific IgG antibody, IL-8 and TNF-α have been found in cerebrospinal fluid (CSF) of cognitively impaired Alzheimer's disease patients infected with *H. pylori*[27,28], and it has been proposed that the inflammatory response induced by *H. pylori* may be involved in the development of Alzheimer's disease. This is consistent with some studies that observed an improvement in parameters of cognitive and functional status and the survival rate of Alzheimer's disease patients after eradication of *H. pylori*[29,30]. However, Alzheimer's disease was independent of *H. pylori* status in a Japanese population[31].

H. pylori-induced chronic atrophic gastritis causes a decrease in serum vitamin B concentration, thereby increasing the concentration of homocysteine[26]. It has been shown that the serum homocysteine concentration correlates with the severity of dementia. Homocysteine-induced oxidative damage has been described in the brain of subjects with mild cognitive impairment, suggesting that oxidative damage may be one of the earliest events in the onset and progression of Alzheimer's disease[30].

GUILLAIN-BARRÉ SYNDROME

Guillain-Barré syndrome is an acute inflammatory autoimmune neuropathy, presenting as a progressive motor weakness usually beginning in the legs, and can be triggered by a preceding bacterial or viral infection. Molecular mimicry of host structures by antigens present in the gastrointestinal pathogens *Campylobacter jejuni* and *H. pylori* are thought to be connected with the development of the autoimmune sequelae observed in Guillain-Barré syndrome[32,33]. In a case-control study, it was found that serum anti-*H. pylori* IgG of patients was significantly higher than that of controls, that CSF anti-*H. pylori* IgG was positive in 80% of patients and in 20% of controls and that the CSF IgG titer was also significantly higher in patients than controls[34]. Furthermore, specific IgG antibodies to vacuolating cytotoxin A (VacA) of *H. pylori* have been detected in the CSF of patients with Guillain-Barré syndrome. The sequence homology found between VacA and human ATPase A subunit suggests that antibodies to VacA bind to ion channels in Schwann cells, resulting in demyelination of motor neurons in these patients[35]. Moreover, high levels of serum anti-*H. pylori* IgG antibodies closely correlate with a more advanced clinical status, and elevation of anti-*H. pylori*-specific IgG antibodies is associated with involvement of the proximal parts of peripheral nerves in patients with acute inflammatory demyelinating polyradiculoneuropathy, the most commonly observed subtype of Guillain-Barré syndrome[32]. Most studies have included only a small sample, so more research is needed to confirm the association between *H. pylori* infection and Guillain-Barré syndrome.

MULTIPLE SCLEROSIS

Multiple sclerosis is the most common inflammatory demyelinating disease of the central nervous system. The association between *H. pylori* infection and multiple sclerosis is a controversial issue and there are few studies that address the problem. *H. pylori* infection is significantly less frequent in patients with conventional multiple sclerosis than in healthy controls or patients with opticospinal multiple sclerosis[36]. However, *H. pylori* infection seems to be one of the risk factors for the development of anti-aquaporin 4 (AQP4) antibody-positive multiple sclerosis, and the eradication of *H. pylori* may be a possible adjunct therapy[37]. Neuromyelitis optica is an inflammatory disease selectively affecting the optic nerves and spinal cord. Chronic persistent infection may contribute to the development of neuromyelitis optica through molecular mimicry between human AQP4 and bacterial AQP. In addition, *H. pylori* neutrophil-activating protein (HP-NAP) contributes to the pathology by inducing migration and activation of neutrophils[37].

ISCHEMIC STROKE

The pathophysiologic mechanism for the majority of

ischemic strokes is occlusion of carotid or cerebral vessels. Infection with *H. pylori* as a risk factor for stroke is still an unresolved issue because of conflicting results. However, a recent meta-analysis showed that chronic infection with *H. pylori* and the presence of CagA-positive strains are statistically significant risk factors for ischemic stroke, especially for noncardioembolic ischemic stroke[38,39]. Similarly, CagA-positive strains of *H. pylori* are significantly associated with atherosclerotic stroke in patients with an active infection[34].

The mechanisms of the high risk for ischemic stroke conferred by chronic *H. pylori* infection are still not understood. It has been hypothesized that *H. pylori* activates platelets and affects coagulation, and it has been shown that six months after eradication of *H. pylori* infection, the plasma levels of total cholesterol, low-density lipoprotein-cholesterol, fibrinogen, and IL-8 were significantly lower than those in *H. pylori*-positive stroke patients and controls[40].

CONCLUSION

Most neurological diseases are the result of a combination of multiple factors, but the systemic inflammatory response and the production of autoantibodies are common components and determinants in the onset, evolution, and outcome of these diseases. Future studies need to focus on determining the molecular mechanisms by which inflammatory mediators induced by *H. pylori* act on the brain, tipping the balance toward a pathological condition.

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TOPIC HIGHLIGHT

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Contemporary review of drug-induced pancreatitis: A different perspective

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Abstract

Although gallstone and alcohol use have been considered the most common causes of acute pancreatitis, hundreds of frequently prescribed medications are associated with this disease state. The true incidence is unknown since there are few population based studies available. The knowledge of drug induced acute pancreatitis is limited by the availability and the quality of the evidence as the majority of data is extrapolated from case reports. Establishing a definitive causal relationship between a drug and acute pancreatitis poses a challenge to clinicians. Several causative agent classification systems are often used to identify the suspected agents. They require regular updates since new drug induced acute pancreatitis cases are reported continuously. In addition, infrequently prescribed medications and herbal medications are often omitted. Furthermore, identification of drug induced acute pancreatitis with new medications often requires accumulation of post market case reports. The unrealistic expectation for a comprehensive list of medications and the multifactorial nature of acute pancreatitis call for a different approach. In this article, we review the potential mechanisms of drug induced acute pancreatitis and provide the perspective of deductive reasoning in order to allow clinicians to identify potential drug induced acute pancreatitis with limited data.

Key words: Drug-induced pancreatitis; Mechanism

Core tip: The knowledge of drug-induced acute pancreatitis (DIAP) is limited by the availability and the quality of the evidence. Potential publication bias may also impact our knowledge of DIAP. Several causative agent classification systems have been proposed, but they require regular updates. In addition, Infrequent prescribed medications and herbal medications are often omitted from those summarized lists. We review the potential mechanisms of DIAP and provide the perspective of deductive reasoning in order to allow clinicians to identify potential DIAP with limited data.

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INTRODUCTION

Acute pancreatitis (AP) is an acute inflammatory condition of the pancreas that may extend to local and distant extra-pancreatic tissues. The annual incidence of AP in the United States is approximately 17 cases per 100000. Acute pancreatitis results in 100000 hospitalizations per year, based on previous reports[1]. An average of 2000 patients per year die from complications related to AP. Although gallstones and alcohol are responsible for more than 90% of all cases in adults, medications have

been recognized as a potential cause of AP[2]. Since the first reported case with chlorthalidone and cortisone in the 1950s, hundreds of commonly prescribed medications from different classes have been reported to induce pancreatic damage. It is expected that the list of drug induced acute pancreatitis (DIAP) will continue to expand with newly approved medications, new cases identified for older agents, and the alternative medicines which have less clinical research support in general. While medications are considered as a common cause of AP, reports of DIAP range from 0.1%-2% of overall cases[2,3].

It is not clear if the true incidence of DIAP has been established due to a lack of mandatory adverse drug report (ADR) system to clinicians, potential publication bias, and the challenge to associate AP with medications. Data from clinical trials of new drugs usually are not informative due to the idiosyncratic character of DIAP. In general, idiosyncratic adverse drug reactions occur with a frequency lower than 1:10000[4]. It is extremely difficult to identify adverse reactions in phase Ⅰ to phase Ⅲ clinical investigational trials. Incretin mimetics have been recently introduced in the treatment of diabetes and are widely used in many countries. Incretin mimetics, including exenatide and sitagliptin, were reported to induce AP shortly after those were approved which resulted in warranted an FDA drug safety communication regarding those agents. However, a meta-analysis of randomized clinical trials of most incretin mimetics including sitagliptin did not show any relevant effect on the incidence of pancreatitis. Overall, the incidence was only 0.1% (22 pancreatitis cases found in a pool of 20312 patients). This is an example of the limitation of clinical trials in finding adverse event with low incidence such as DIAP[5].

The knowledge of DIAP is also limited by the availability and the quality of the evidence. It can be difficult to rule out other causes of DIAP, especially in patients who have multiple comorbidities, medications, and underlying risk factors. Since all reports depend on the judgment of the clinicians to exclude other possible causes, reporting more severe ADR has also lead to publication bias. Due to its rarity, most of the evidence comes from case reports of individual drugs and few from case control studies. With a lack of standard ADR reporting format, inadequate data collection in several domains, such as the drug dose, onset of DIAP relative to the use of the medication, and exclusions of other causes, makes it difficult to establish a true causality. In addition, a causal relationship between the agents and DIAP may be difficult to establish due to ethical and practical considerations of re-challenge with the suspected agents. Therefore, the definite relationship between DIAP and medications has only been established in no more than 6% of the agents that have been shown to cause DIAP[6]. Since the identification of DIAP has relied mostly on individual case reports, specific drugs instead of the entire class are usually noted, which makes it even more challenging to identify possible cases in a timely manner.

Potential publication bias may also impact our understanding of DIAP and influence how DIAP is being managed. New drugs or medications with known severe side effects are usually more closely monitored than those that have been in existence for a long time, infrequently prescribed, or considered harmless (*i.e.*, over the counter medications or herbal supplements). Despite the low incidence of drug-induced AP, it is associated with higher morbidity, extended hospital stays, and increased healthcare cost[7]. Approximately 25% of the cases may require intensive care treatment[8]. Developing a systemic approach of identifying potential DIAP is warranted. The aim of this review is to offer a different perspective of approaching DIAP by examining the potential mechanisms of DIAP in order to allow clinicians to identity possible cases with limited data.

ETIOLOGY OF ACUTE PANCREATITIS

Acute pancreatitis is an inflammatory process of the pancreas with varying involvement of other regional tissues or remote organ systems. Gallstone and alcohol use have been considered the most common causes of acute pancreatitis. Gallstone-associated AP is mainly identified by imaging. Previous association with tobacco use is directly linked to alcohol abuse. More evidence associates tobacco use as another toxin that can be directly linked to both acute and recurrent pancreatitis. Other potential mechanical etiologies include periampullary pathologies including intraductal tumor or parasites that are possible in developing countries. In addition to the most common causes, other etiological risk factors for acute pancreatitis are associated with mechanical factors including pancreas divisum, endoscopic retrograde cholangiopancreatography and manometry, as well as trauma or surgical procedures near the pancreas.

Metabolic or systemic process such as hyperlipidemia, infection, and chronic hypercalcemia are well known causes of pancreatitis as well[9]. Infections and toxins, including viral etiologies: mumps, coxsackievirus, hepatitis B, cytomegalovirus, varicella-zoster, herpes simplex virus, human immunodeficiency virus. Bacteria such as *Mycoplasma*, *Legionella*, *Leptospira*, *Salmonella*, *Aspergillus*, *Toxoplasma*, *Cryptosporidium* and *Ascaris* are potential causes of AP. Last, but not least, vascular diseases and pregnancy are also described as causes for pancreatitis.

AP can occur if there is damage to the acinar cells and/or injury to the pancreatic duct that leads to inappropriate accumulation and activation of proenzymes within the pancreas. The activated pancreatic enzymes digest the cell membranes of the pancreas and activate an inflammatory response, which increases the vascular permeability of the pancreas. Hemorrhage, edema, ischemia, and necrosis can result[1,9]. Data from animal studies show that reduced exocytosis and premature fusion of zymogen granules to lysosomes in pancreatic exocrine cells may activate pancreatic proenzymes and lead to cellular autodigestion.

Table 1 Classification system of drug-induced acute pancreatitis according to Badalov *et al*[11]

	Definition	Example
Class Ⅰ drug	Ia: at least one case report, evidence of a positive re-challenge, and exclusion of other causes of AP	Codeine, cytarabine, dapsone, enalapril, furosemide, isoniazid, mesalamine, metronidazole, pentamidine, pravastatin, procainamide, simvastatin, sulfamethoxazole, sulindac, tetracycline, valproic acid
	Ib: similar to class Ia, except that other causes of AP could not be ruled out	Amiodarone, azathioprine, dexamethasone, ifosfaide, lamivudine, losartan, 6-MP, premarin, TMP-SMZ
Class Ⅱ drugs	Include at least four case reports with a consistent latency period for at least 75% of the cases	Acetaminophen, Clozapine, DDI, erythromycin, estrogen, l-asparaginase, propofol, tamoxifen
Class Ⅲ drug	At least two case reports but do not have re-challenge data or a consistent latency period	Alendronate, carbamazepine, ceftriaxone, clarithromycin, cyclosporin, hydrochlorothiazide, interferone/ribavirin, metformin, minocycline, naproxen, paclitaxel, prednisone, prednisolone
Class Ⅳ drug	One case report without re-challenge data	Ampicillin, cisplatin, colchicine, cyclophosphamide, diclofenac, doxorubicin, interleukin-2, octreotide, propoxyphene, rifampin, risperidone, sertaline, tacrolimus, vincristine

AP: Acute pancreatitis; 6-MP: 6-mercaptopurine; TMP-SMZ: Trimethoprim and sulfamethoxazole.

CLASSIFICATION OF CAUSATIVE AGENTS

It is difficult to determine if the effects are intrinsic for all members of a drug class despite reports of DIAP incidence within the class. Several classification systems have been proposed. A substantial number of medications are known to cause AP, however, the underlying mechanism is still not well understood. The classification systems rely on summarized lists of medications from previously published reviews to help make the diagnosis of DIAP. Mallory and Kern in 1980s classified drugs that may cause pancreatitis into three groups: definite, probable, or possible association with pancreatitis[5,9]. In order to improve the quality of evidence, different classification systems have also been proposed that categorized DIAP in classes based on the number of reports and re-challenge results[5,10].

Badalov *et al*[11] in 2007 expanded the classification system to five categories: Ⅰa, Ⅰb, Ⅱ, Ⅲ, and Ⅳ (Table 1). Classifications are based on the published reports from 1955 to 2005. Class Ⅰa includes drugs with at least one case report, evidence of a positive re-challenge, and exclusion of other causes of AP. Class Ⅰb is similar to class Ⅰa, except that other causes of AP could not be ruled out. Criteria for class Ⅱ drugs include at least four case reports with a consistent latency period for at least 75% of the cases. Class Ⅲ drugs have at least two case reports but do not have re-challenge data or a consistent latency period. Finally, class Ⅳ drugs have one case report without re-challenge data. This classification provides a quick reference of potential causative agents based on the available data at the time of the review. However, regular updates of existing classification are needed since new cases of DIAP are reported continuously. Furthermore, infrequently prescribed medications and alternative medications are often omitted from these summarized lists.

Mechanism of DIAP

The majority of the reported DIAP cases seem to have an idiosyncratic character. Idiosyncratic reactions to drugs are adverse effects that are not directly related to pharmacodynamic mechanisms of the drugs. These adverse events can occur unpredictably via abnormal interactions between the drugs and the organism, which is usually mediated by immunologic or cytotoxic effects triggered by the drug or its metabolites in a specific organ, in this case, the pancreas[11]. Although the exact mechanism of DIAP is not always known, the pathogenesis should not differ from other causes of AP. It is believed that the pathogenesis of AP differs only in the injury mechanism. It consists of three steps: (1) premature activation of trypsin in acinar cells; (2) intrapancreatic inflammation; and (3) extrapancreatic inflammation[5]. Several mechanisms have been hypothesized including immune-mediated, direct pancreatic toxicity, pancreatic-duct constriction, influence of medication on the bile flow, thrombosis, metabolic effects, and hypersensitivity[12,13]. Mechanisms of DIAP is showed in Figure 1.

Researchers have also used latency to classify the potential mechanisms of DIAP[5]. It is hypothesized that direct immunological effects are usually observed within the first month of drug exposure, whereas toxic effects are noted after a few months of treatment. Potential mechanisms of DIAP include hypersensitivity (onset after four to eight weeks of use), accumulation of a toxic metabolite (onset after several months of use), hypertriglyceridemia (onset after several months of use), and intrinsic toxicity, which is sometimes related to overdose (onset may be almost immediate)[14]. However, there are exceptions that exist. Clinicians should not ignore long lasting medications while DIAP is a concern.

The following reviews summarize five major types of mechanisms of DIAP (Table 2), namely structural, toxins, metabolic, vascular, and other.

Structural

The structural damage such as compression, obstruction, or inflammation of the pancreatic duct may lead to AP. The most common cause for obstruction is choledochlithiasis, or gallstones. Obstruction can also be caused by

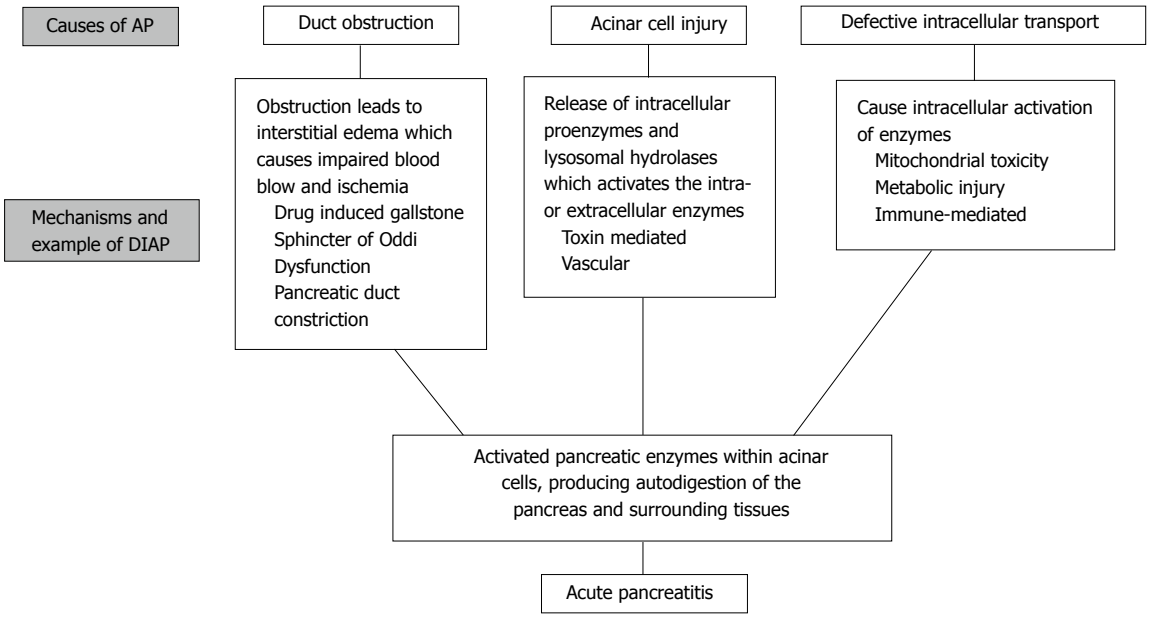

Figure 1 Mechanism of drug-induced pancreatitis. AP: Acute pancreatitis; DIAP: Drug-induced acute pancreatitis.

Table 2 Mechanism of drug induced pancreatitis with drugs associated with acute pancreatitis

Mechanism of DIAP	Drugs with a definite relationship or with class I / II to AP	Probable	Similar structure/class/mechanism with reported cases
Structural	Cholestatic liver injury		Rofecoxib
	Azathioprine		
	Cytarabine		
	Spasm of the sphincter of Oddi	Octreotide	Opium
	Opioids		Marcolides
	Codeine		
	Erythromycin		
	Obstruction		ACE-inhibitors
	Enalapril-angioedema		
	Duct constriction		NSAIDs
	Sulindac		
	Stone		Ceftriaxone
			Dipyridamole
Toxins	Acetaminophen	Metformin	Minocycline
	Didanosine		Tigecycline
	Isoniazid		Doxycycline
	Metronidazole		NRTI
	Valproic acid		HMG-CoA reductase inhibitors
	Mesalamine		
	Pentamidine		
	Asparaginase		
	Sitaliptin		
	Exenatide		
	Tetracycline		
	Pravastatin		
Metabolic	Hypertriglyceridemia	Hydrochlorothiazide	Isotretinoin
	Estrogens	Interferon alfa	Retinoid derivaties
	Corticosteroids	Propofol	Protease inhibitors
	Furosemide	Tamoxifen	Saw palmetto
	β-blocker		Ethacrynic acid
	Clomiphene		Anti-psychotics (aripiprazole, clozapine, olanzapine, quetiapine, risperidone)
	Hypercalcemia		Ⅳ calcium
			Vitamin D
Vascular			Contrast media - iopamidol Procainamide
Immune-mediated	Azathioprine/mercaptopurine sulfasalazine		

AP: Acute pancreatitis; NRTI: Nucleoside reverse transcriptase inhibitor; NSAIDs: Nonsteroidal anti-inflammatory drugs.

duodenal inflammation in Crohn's disease[1].

Medications with risk of gallstones: Ceftriaxone, a third-generation cephalosporin that is excreted from bile duct, has been associated with the development of sludge or stones in the gallbladders for some patients treated with this medication. Secondary pancreatitis has been considered in association with ceftriaxone-induced pseudolithiasis[15]. Unlike ceftriaxone, the kidney pathway is the major means of elimination for most of cephalosporins. It could potentially explain why DIAP has not been reported as class wide induced disease.

Based on an increased amount of cholesterol secreted in bile, causing an increased risk of gallstones which may explain the mechanism of 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitor-induced AP[10]. Long-term administration of dipyridamole and octreotide can form insoluble substances that precipitate in the gallbladder bile to promote gallstone formation as they are highly excreted from the bile[16,17].

Medications that Cause Sphincter of Oddi Dysfunction: As another example of structural disturbance, the Sphincter of Oddi (SO) is situated at the junction of the bile and pancreatic ducts where they enter the duodenum and serves to regulate the flow of bile and pancreatic juices as well as preventing reflux of duodenal contents into the pancreatobiliary system. SO dysfunction refers to two possible conditions-papillary stenosis (edema or hypertrophy) and dyskinesia (tachyoddia, induced spasm) that lead to partial or complete obstruction of the pancreatic duct resulting in pancreatitis[18]. SO dysfunction is implicated as a cause of various forms of AP including gallstone pancreatitis, pancreatitis secondary to alcohol, scorpion envenomation, and organophosphate poisoning. Medications such as octreotide, opioids, opium, and codeine reportedly induce AP in association with SO dysfunction[19,20]. Erythromycin can cause DIAP due to its prokinetic effect on the smooth muscle of the gastrointestinal track and the gallbladder subsequently increasing the pressure of the SO[21]. Since all macrolide antibiotics have prokinetic effect of different degrees, it is reasonable to consider that AP could potentially be drug related when patients are treated with these agents. As an example, clarithromycin and azithromycin have been reported to be associated with AP[6].

The mechanism of action of the class of drug is also an important factor when evaluating the relatedness of the adverse event to the drug. The probable mechanism of aspirin- or nonsteroidal anti-inflammatory drug (NSAID)- induced pancreatitis is due to inhibition of prostaglandins that otherwise may cause pancreatic duct constriction[12]. Aspirin is shown to increase pancreatic duct permeability in animal models. It increases calcium secretion from the pancreas, which is considered a marker of pancreatic damage. Experimental studies suggested that prostaglandins may have a protective effect on pancreatic cells[22]. Membrane stabilization of pancreatic cells may be the mechanism behind the cytoprotection conferred by prostaglandins. In NSAID-associated AP, sulindac seems to stand out as the individual drug from the class with the highest number of published cases[23-26].

Toxins

Cumulative dose-dependent effect of toxic metabolites is also hypothesized in drugs showing a consistent long latency (more than 30 d) at the onset of the first episode of DIAP such as valproic acid[5]. Below we discussed a few classic examples of toxin-mediated DIAP.

Nucleoside reverse transcriptase inhibitor: The leading hypothesis of nucleoside reverse transcriptase inhibitor (NRTI)-associated pancreatitis involves mitochondrial toxicity caused by the inhibition of human mitochondrial DNA polymerase-gamma[27]. This inhibition leads to impaired oxidative phosphorylation and failure to synthesize ATP, which is vital for energy-requiring reactions within the cell. Tissues with the highest energy demand appear to be most susceptible. Mitochondrial toxicity is shared among nucleoside analogues and AP attributed to these agents has been described[28]. The degree of mitochondrial impairment and the resultant tissue-specific clinical manifestations vary depending on the NRTI. Among non-nucleoside reverse transcriptase inhibitors, nevirapine is associated with pancreas-related toxicities, whereas efavirenz is not[29,30].

Metronidazole: One speculative mechanism of metronidazole-induced pancreatitis is that under aerobic conditions, it may undergo redox cycling and yield hydrogen peroxide, superoxide, and other free radicals, which can be toxic to pancreatic beta cells and induce pancreatitis[31].

Pentamidine: Pentamidine has a cytotoxic effect on pancreatic β-cells isle and can cause hypoglycemia or hyperglycemia[32]. Same effect can be expected on acinar pancreatic cells.

L-asparaginase: Animal models suggested that L-asparaginase -induced pancreatic injury can involve disruption of the plasma amino acid balance. Disruption of protein synthesis in acinar cells can cause inhibition of exocytosis following the histologic morphologic changes[33].

Tetracycline: Medications in the tetracycline class, including tetracycline, minocycline, and oxytetracycline, are also associated with AP[34-37]. Tetracycline-induced fatty metamorphosis of the liver usually accompanies evidence of pancreatitis, but pancreatitis without evidence of liver disease has also been observed after administration of tetracycline. Steinberg hypothesized that accumulation of an unidentified toxic metabolite may be the cause of tetracycline-induced pancreatitis[38]. Others suggest that high biliary concentration of tetracycline may be associated with tetracycline-induced pancreatitis[39]. Bile concentrations of minocycline after a 200 mg loading dose followed

by one single 100 mg dose were observed to be more than 10 times higher than concurrent serum concentrations (mean serum concentration 0.65 mcg/mL, range 0.07-1.85 mcg/mL)[40]. Tigecycline, the first available member of the glycylcycline group, is a derivate of minocycline and can share similar side effects. Concentrations of tigecycline in bile (median 75.2 mg/L, range 15.9-1150 mg/L) were also found to be several logs greater than concurrent serum concentration (median 0.112 mg/L, range 0.042-0.25 mg/L) after a single 100 mg dose. The mean and median bile-to-serum 24-h area under the concentration-time curve (AUC0-24) ratios were 537 and 368 respectively[41]. It is reasonable to suspect DIAP as a possible complication in tigecycline treated patients. Several cases have been reported previously[42].

Metabolic

Hypertriglyceridemia: It is generally accepted that levels of triglycerides (TG) greater than 1000 mg/dL may increase the risk of precipitating an episode of pancreatitis[43]. The breakdown products of TG are probably responsible for inducing pancreatitis. When lipase in the pancreatic capillary bed acts on the high levels of TG in serum, toxic free fatty acids are generated. The endothelial lining of small pancreatic blood vessels is the first site of injury. Damages of small blood vessels lead to recruitment of inflammatory cells and thrombosis. Hyperlipidemic pancreatitis may be associated with normal serum amylase but with elevated serum lipase levels[44]. With excessive TG, local ischemia and acidemia may occur due to capillary obstruction[45]. This damage exposes TG to pancreatic lipases, which impact degradation of TG[46]. Hydrolysis of TG by pancreatic lipase, excessive formation of free fatty acids with inflammatory changes, capillary injury, and hyperviscosity are postulated to account for the development of hypertriglyceridemia-induced pancreatitis.

Drugs including estrogens, isotretinoin, propofol, retinoid derivatives, HIV protease inhibitors, β-blockers, thiazides, and furosemide are thought to induce AP owing to hypertriglyceridemia. Estrogen is the most well studied drug in this manner. Exogenous estrogens increase serum TG and fatty acids primarily by reducing levels of lipoprotein and hepatic lipases, which subsequently decrease clearance and aggravate insulin resistance[47]. Typically, estrogen-related pancreatitis occurs within the first months following estrogen initiation. Obese patients with underlying glucose intolerance or fasting hypertriglyceridemia are at greater risk[44]. However, reports have also shown that estrogen-associated DIAP can happen without elevated serum lipid concentrations[48,49]. It is thought that arteriolar thrombosis may be another potential mechanism of action[48,50]. Tamoxifen and clomiphene are synthetic estrogen analogues with mixed agonist-antagonist actions. Cases of tamoxifen- or clomiphene-associated AP have been reported with mechanisms similar to that of estrogen.

Dibenzodiazepine-derived atypical antipsychotics (*i.e.*, clozapine, olanzapine, and quetiapine) may also be a potential cause of DIAP. Both risperidone and ziprasidone are non-dibenzodiazepine atypical antipsychotics and appear to have minimal effect on serum lipids[51]. This is another example where clinicians can apply the general knowledge of each medication when evaluating the likelihood of DIAP for the newer medications.

The previous section discussed that tetracyclines-associated DIAP due to its toxic metabolite and high biliary concentrations. Elmore and Rogge[36] also proposed a tetracycline-induced hypertriglyceridemia mechanism with subsequent pancreatitis. Tetracycline inhibits protein synthesis by binding to the 30S ribosomal subunit in the messenger ribonucleic acid (mRNA) translation complex. Blockage of protein synthesis could result in accumulation of defective proteins within hepatocytes. This inhibits the release of TG from the liver, which may lead to pancreatitis.

Hypercalcemia: Calcium is identified as the most important intracellular element in acinar cell stimulus-secretion coupling[52]. Disruption in the secretory process could be the mechanism by which hypercalcemia induces pancreatitis. Based on experimental studies, increase in extracellular calcium leads to a functional secretory block with dose-dependent characteristics[53]. Acinar cell stimulation induces spikes in cytosolic calcium concentration by repetitively releasing calcium from intracellular stores, which activates the normal secretory process of digestive enzymes from intracellular zymogen stores. Excessive extracellular calcium concentration leads to sustained increases in cytosolic calcium. It results in vacuole formation and trypsinogen activation and eventually leads to edematous or necrotizing pancreatitis[54]. Research indicates that hypercalcemia is associated with an increase in serum enzymes[44]. Intravenous calcium administration has been associated with pancreatitis in at least two published reports. Additionally, pancreatitis has been correlated to cases of vitamin D poisoning and to patients receiving total parenteral nutrition[55]. It is suspected that all drugs which can cause hypercalcemia carry risk of inducing AP.

Thiazides, a class with hypertriglyceridemia potential, could also induce hypercalcemia and hypophosphatemia. Thiazide-induced reductions in blood pressure may lead to pancreatic ischemia. They may act directly on the pancreas or indirectly by altering calcium metabolism. Therefore, there are multiple mechanisms exhibited by thiazides that could potentially lead to AP.

Vascular

Ischemia is an uncommon cause of AP. Pancreatic infarcts may occur in patients with underlying atherosclerotic vascular disease, but they are unusual because the pancreas is richly perfused from several different arterial sources. Cholesterol emboli may cause pancreatitis, cholecystitis, or bowel ulceration or infarction, and should be suspected when AP occurs after vascular interventions such as cardiac catheterization. Patients may have associated evidence

of renal, gut, or peripheral cholesterol emboli. Ischemic pancreatic and hepatic injury may be associated with malignant hypertension, low flow states due to severe heart failure, or administration of potent vasoconstrictors. Vasculitis may cause pancreatitis associated with systemic autoimmune diseases. Acute pancreatitis secondary to drug-induced lupus syndrome has also been described[56].

Contrast-induced pancreatitis may be related to decreased oxygenation and impaired circulation of the pancreas. Iopamidol has a viscosity of 9.4 cP at 37 degrees centigrade versus human plasma of 1.72 cP at hematocrit of 43%. A similar pathophysiologic process has been proposed in contrast-induced kidney injury. Cholesterol crystal embolization may be another mechanism that results in occlusion of small arteries[57].

Immune-mediated reaction

Direct immunological effects are usually observed within the first month of drug exposure, whereas toxic effects are noted after a few months of treatment[45]. Researchers have also considered AP an immune-mediated reaction if relapse occurs rapidly after re-challenge as seen with sulfonamides and aminosalicylates (*e.g.*, sulfasalazine and mesalazine)[58-60]. The latency between initiation of the drug and the onset of DIAP is usually one week to a month, but reexposure can lead to a new episode in one to three days[5]. Cases of azathioprine or the thiopurine bases mercaptopurine-induced pancreatitis are well documented. Studies have shown patients with decreased levels of thiopurine metabolizing enzyme inosine triphosphate pyrophosphatase may be at an increased risk of developing thiopurine-induced AP. However, 6-tioguanine-induced pancreatitis is less common than conventional thiopurine. Only 1% of inflammatory bowel disease (IBD) patients previously intolerant to the conventional thiopurines are reported to have 6-tioguanine-induced AP after treatment[61]. A strong correlation with immune disorders, mainly Crohn's disease and HIV infections, implies an immune-mediated reaction as a chief causative factor of the disease.

Angiotensin-converting-enzyme inhibitors: Captopril, enalapril, lisinopril, perindopril, benazepril, and quinapril have all been associated with AP[25,52]. Pancreatic duct obstruction by local angioedema may be the mechanism by which angiotensin-converting-enzyme-inhibitors cause pancreatitis. Others propose a direct toxic effect on pancreatic cells. Since captopril is structurally dissimilar to enalapril and lisinopril, an allergic reaction seems less likely. Angiotensin receptor blockers may share a similar mechanism for pancreatitis, at this point definitive cases are not described in literature[10].

Alternative medicines including herbal medication

There are very limited data of DIAP associated with herbal or over the counter medications when compared to prescription medications. Although a mechanism for saw palmetto-induced AP has not been thoroughly established, cases of saw palmetto-induced cholestatic hepatitis associated with AP have been reported. Currently, there are only two reported cases of saw palmetto induced AP. Another theory suggests that it occurs through its estrogenic effects by stimulating estrogen receptors and then induces a hypercoagulable state that leads to pancreatic necrosis[2]. This information should prompt clinicians to consider saw palmetto a potential cause of AP.

A WORK UP FOR ACUTE PANCREATITIS

Since no specific test for establishing the diagnosis of DIAP is available, the diagnosis is usually based on excluding all other common causes. Pancreatitis is suspected when a patient presents with clinical features including acute onset of persistent and severe epigastric abdominal pain, and is then confirmed by laboratory and imaging studies that exclude other serious intra-abdominal conditions. Most patients will have elevations in serum levels of amylase or lipase within a few hours of the onset of symptoms. Lipase tends to remain elevated longer than amylase. Amylase and lipase levels above three times the upper limit of normal are mostly associated with pancreatitis. Once these levels are elevated, serial measurements are of no clinical significance for prognosis or outcomes. They should not be obtained routinely after the initial measurements are obtained.

Imaging studies are used to establish the diagnosis, but also to determine etiology and prognosis. Both abdominal ultrasound and abdominal computed tomography (CT) can be used interchangeably; however, the latter is preferred as it can provide alternative diagnoses.

As part of the investigation for potential causes of AP, a history of alcohol/tobacco use, previous biliary colic, medication history, family history, and recent trauma should be elicited. Gallstone-associated pancreatitis should be suspected if stones are seen on imaging studies or if liver chemistries are abnormal and then improve over a few days. A three-fold elevation of ALT has a high predictive value for gallstone-associated AP. Hypertriglyceridemia, especially with levels above 1000 mg/dL, and hypercalcemia can be evaluated based on laboratory data. Infections, including viral etiologies, are potential causes of AP as well.

A suspected drug etiology should be considered after the exclusion of more common causes of illness. As mentioned above, it is challenging to establish the causality between medications and associated AP. The use of classification systems may be useful as the first screening tool. Since the mean interval between initial drug administration and start of the symptoms is approximately 5 wk, with a range of 2 to 36 wk[46], clinicians can target those medications when reviewing the medication profiles. If the mediations are not listed on these summarized lists, clinicians should identify if similar structured medications have been associated with DIAP and evaluate the possibility of sharing a similar mechanism of inducing pancreatitis. Once the target agent is identified, the offending agent

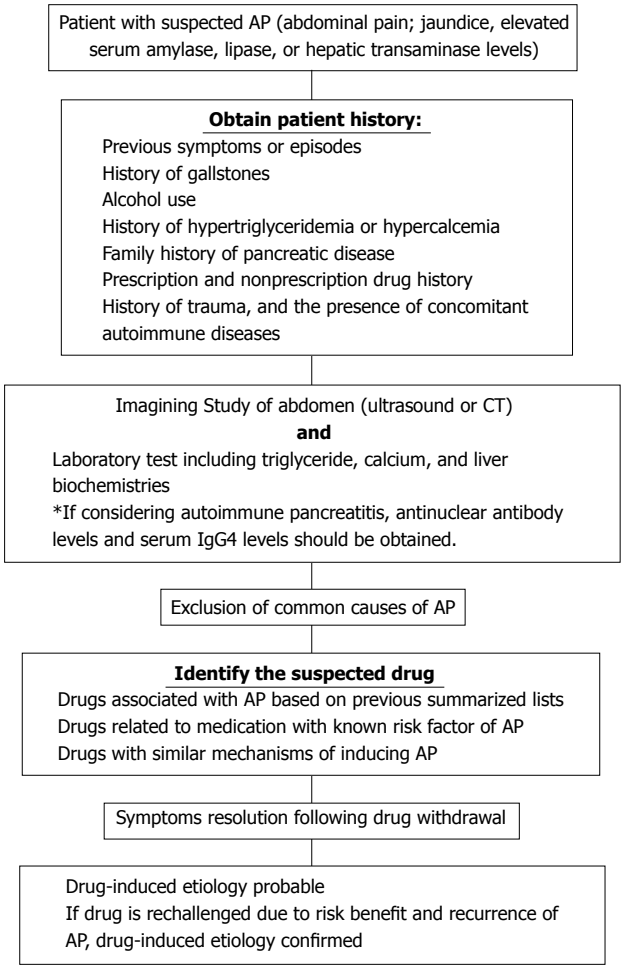

Figure 2 Algorithm of Identifying A potential case of drug induced acute pancreatitis. AP: Acute pancreatitis.

should be discontinued, preferably one at the time to avoid confounders. Most reactions are reversible and resolve on their own within 3-7 d after the offending agent has been discontinued. Due to the nature of the disease state and ethical consideration, re-challenge of the suspected drug is usually not possible. Often times, the medication can be just a possible/probable cause of AP. If re-challenge of the suspect drug is considered necessary, the patient's written informed consent should be obtained. An algorithm of identifying a potential case of drug induced AP is presented in Figure 2.

DISCUSSION

Hundreds of medications have been suggested to be the potential cause of AP, although the true incidence of DIAP is unknown. Evidence associating drugs with AP is largely based on individual cases. It is unrealistic to expect a comprehensive list that includes all agents associated with AP due to continuously reported new cases. Although relapse of pancreatitis after controlled re-challenge confirms a causal relationship, such proof is uncommon. Furthermore, re-challenge is only ethical when the same treatment is absolutely necessary for the patient. It leads to only few causal relationships for the reported agents.

Few causative agent classifications have been proposed. These classifications have helped clinicians understand the quality of evidence behind each potential causative agent. However, with the exception of a few agents with a definite relationship confirmed by re-challenge, it depends on each individual report to exclude all other possible causes, especially drug effects that may be difficult to separate from the underlying conditions. Clinically, certain subpopulations such as children, women, the elderly, patients with IBD and patients with HIV appear to be at a higher risk[2]. Mesalazine, azathioprine, and corticosteroids, for instance, are used in the treatment of IBD which itself increases the risk of AP. Anti-retroviral agents is another example as HIV is an independent risk factor of AP. A study that compared patients with and without HIV infection found a drug-related etiology in 41% and 5% of the patients with AP respectively[62]. The use of NRTIs such as didanosine, stavudine, and lamivudine and co-administration of other medications such as pentamidine, cotrimoxazole, antimycobacterial therapy, or cytotoxic chemotherapy for at least 6 mo were found to be a significant risk factor for at least a three-fold increase in serum pancreatic enzymes ($P < 0.05$). Certain medications, such as proton pump inhibitors and histamine2-receptor antagonists as well as NSAIDs, may be initiated in response to early symptoms of unrecognized pancreatitis. This may have led to erroneously attributing the pancreatitis to these medications[63,64]. Repeated cases of DIAP are more likely to be published or even diagnosed than those without prior reports. Due to underreporting incidence rates from spontaneous reports and potential publication bias with only reporting severe cases, it has further complicated the assessment of the causal relationship between drugs and AP based on current proposed classification.

Efforts have been devoted to improve drug safety surveillance strategies. Vilar *et al*[65] have shown promising results of detecting adverse drug events related to pancreatitis by developing molecular fingerprint-based models. The models were based on the premise that similar molecules can have comparable biological properties. For example, tigecycline is structurally related to minocycline and shares similar pharmacokinetic properties and side effects with tetracyclines. Not surprisingly, cases of tigecycline-induced AP were reported soon after its introduction to the market[42]. Nevertheless, DIAP is generally not considered as a drug class effect, so specific drugs are usually noted instead of the entire class[14]. It is suggested that clinicians take the potential mechanism of DIAP into account. For example, ceftriaxone has different pharmacokinetic properties than other cephalosporins and may lead to secondary pancreatitis caused by only ceftriaxone induced pseudolithiasis.

Limited data exist regarding the mechanisms of DIAP. The pathogenesis is not completely understood. Nevertheless, DIAP should not have unique features that

distinguish it from AP due to other causes. Drugs may lead to pancreatitis by inducing known risk factors of AP such as structural (*e.g.*, cholestatic liver injury, spasm of the SO, duct obstruction/constriction, and stones), metabolic (*e.g.*, hypertriglyceridemia and hypercalcemia), and vascular effects. Some drugs or drug metabolites may theoretically have a direct toxic effect on the pancreas. Other than known mechanisms of toxicity such as mitochondrial toxicity and protein synthesis inhibition, the high level of gastrointestinal drug concentration may be needed to cause cytotoxic damage. Drugs with a definite causal relationship to AP including isoniazid, metronidazole, valproic acid, mesalamine, and tetracycline share similar pharmacokinetic properties by extensive hepatic metabolism. If other potential causes of DIAP have been ruled out, drugs that are highly concentrated in the gastrointestinal tract could be potential suspects of DIAP. For other drugs, an immunoallergic idiosyncratic reaction is more likely. Re-challenge with these drugs usually leads to prompt recurrence of symptoms in a dose-independent manner. In an animal study, the results suggest that DIAP is multifactorial and may explain why the incidence of DIAP is low[18].

Establishing a definitive causal relationship between a drug and AP poses a challenge to clinicians. Depending on the agents, the time from the initiation of therapy to the onset of pancreatitis symptoms varies. Pancreatitis can occur within a short time after administration of the first dose to years after therapy begins for most of the drugs. The unrealistic expectation of the comprehensive list and the multifactorial natures of the causes of AP call for a different approach. This article reviews the potential mechanisms of DIAP and provides the perspective of deductive reasoning in order to identify potential DIAP.

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TOPIC HIGHLIGHT

WJGP 5th Anniversary Special Issues (3): Pancreatitis

Acute pancreatitis in children and adolescents

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Abstract

In this Topic Highlight, the causes, diagnosis, and treatment of acute pancreatitis in children are discussed. Acute pancreatitis should be considered during the differential diagnosis of abdominal pain in children and requires prompt treatment because it may become life-threatening. The etiology, clinical manifestations, and course of acute pancreatitis in children are often different than in adults. Therefore, the specific features of acute pancreatitis in children must be considered. The etiology of acute pancreatitis in children is often drugs, infections, trauma, or anatomic abnormalities. Diagnosis is based on clinical symptoms (such as abdominal pain and vomiting), serum pancreatic enzyme levels, and imaging studies. Several scoring systems have been proposed for the assessment of severity, which is useful for selecting treatments and predicting prognosis. The basic pathogenesis of acute pancreatitis does not greatly differ between adults and children, and the treatments for adults and children are similar. In large part, our understanding of the pathology, optimal treatment, assessment of severity, and outcome of acute pancreatitis in children is taken from the adult literature. However, we often find that the common management of adult pancreatitis is difficult to apply to children. With advances in diagnostic techniques and treatment methods, severe acute pancreatitis in children is becoming better understood and more controllable.

Key words: Acute pancreatitis; Children; Pathophysiology; Etiology; Diagnosis; Treatment

Core tip: The etiology, manifestations, and course of acute pancreatitis in children are often different than in adults, and these differences should be highlighted. The etiology of acute pancreatitis in children is drugs, infections, trauma, or anatomic abnormalities. The diagnosis of acute pancreatitis is based on clinical symptoms, serum pancreatic enzyme levels, and imaging studies. Treatments in adults and children are similar. With advances in diagnostic techniques and treatments, severe acute pancreatitis in children is becoming better understood and more controllable.

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INTRODUCTION

Acute pancreatitis is not necessarily a rare disease, even in children and adolescents (hereinafter referred to as "children"), and may be life-threatening if it is severe[1,2]. Therefore, acute pancreatitis should always be considered during the differential diagnosis of abdominal pain in children, and appropriate treatment should be started promptly when necessary. However, many treatment regimens are based on consensus conferences and evidence in adults, so a search for the cause and appropriate treatment in children is often difficult[3,4]. This paper discusses the causes, diagnosis, and treatment of acute pancreatitis in children, including a review based on our own experiences.

Table 1 Etiology of childhood acute pancreatitis

Congenital anomalies, periampullary obstruction
Choledochal cyst, abnormal union of the pancreaticobiliary junction, gallstone, cholecystitis, pancreatic divisum, tumor, ascaris aberrant
Infectious
Mumps, measles, coxsackie, echo, lota, influenza, epstein-barr virus, Mycoplasma, salmonella, gram-negative bacteria
Drugs
L-asparaginase, steroid, valproic acid, azathioprine, Mercaptopurine, mesalazine, Cytarabine, Salicylic acid, indomethacin, tetracycline, chlorothiazide, isoniazid, anticoagulant drug, borate, alcohol
Trauma
Blunt injury, child abuse, ERCR, After surgery
Systemic disease
Reye syndrom, systemic lupus erythematosus, polyarteritis nodosa, Juvenile rheumatoid arthritis, sepsis, multiple organ failure, Organ transplantation, hemolytic-uremic syndrome, henoch-schoenlein purpura, kawasaki disease, inflammatory bowel disease, chronic intestinal pseudo-obstruction, gastric ulcer, anorexia nervosa, food allergy, cystic fibrosis
Metabolic
Hyperlipoproteinemia (I, IV, V), hypercalcemia, diabetes, α1 antitrypsin deficiency
Nutrition
Malnutrition, high-calorie infusion, vitamin A and D deficiency
Others
Familial, idiopathic

ERCP: Endoscopic retrograde cholangiopancreatography.

Table 2 Cause of acute pancreatitis in children and adolescents

Ref.	Location	Cases	Etiology (%) Systemic	Biliary[1]	Anatomic[2]	Trauma	Familial	Metabolic[3]	Drugs	Others[4]	Idiopathic
Lopez[50]	United States	274	48	10	NA	19	NA	0.7	5	0.4	17
DeBanto *et al*[1]	United States	301	3.5	10.5	1.5	13.5	5.5	4	11	16.5	34
Werlin *et al*[8]	United States	180	14	12	7.5	14	3	5.5	12	24	8
Nydegger *et al*[4]	Australia	279	22.2	5.4	NA	36.3	NA	5.8	3.2	2.2	25.1
Suzuki *et al*[19]	Japan	135	8.9	30.4	25.9	9.6	NA	NA	11.1	3.7	10.4
Lantz *et al*[2]	United States	211	3.3	11.8	5.2	7.6	0.9	6.2	19.9	13.8	31.3

All studies contained more than 100 cases. NA: Not available. [1]Gallstone, biliary sludge, choledochal cyst; [2]Abnormal union of the pancreaticobiliary junction, pancreatic divisum; [3]Diabetic acidosis, hyperlipidemia, organic acidemias, hypercalcemia; [4]Associated viral infection, postendoscopic retrograde cholangiopancreatography, alcohol, autoimmune, cystic fibrosis, post-surgery.

ETIOLOGY

Alcohol and gallstones are the etiology of acute pancreatitis in many adults, and although some differences exist based on sex and ethnicity, these two etiologies account for more than 60% of cases of acute pancreatitis in adults[5,6]. However, the etiology in children is often drugs, infections, trauma, and anatomic anomalies such as choledochal cysts and abnormal union of the pancreatobiliary junction (Table 1)[1,4,7,8]. Table 2 shows the incidence of acute pancreatitis by etiology. There is a considerable difference in the etiology of acute pancreatitis in Western and Asian children[9].

Drugs

Among drugs used in childhood and adolescence, L-asparaginase (ASNase), steroids, and valproic acid often cause pancreatitis as an adverse reaction. In particular, ASNase, a key drug used in treatment of childhood leukemia, is associated with a higher incidence of pancreatitis as compared to other drugs, ranging from 2%-16% when mild cases are included[10-12]. A characteristic of pancreatitis associated with ASNase, in addition to clinical symptoms of abdominal pain and tenderness, is the early absence of elevated serum amylase levels in about half of patients[13,14]. This phenomenon is attributed to inhibition of protein synthesis by ASNase[14]. Therefore, when acute pancreatitis is suspected based on clinical findings, even in the absence of serum amylase elevation, acute pancreatitis must always be considered in the differential diagnosis, and it is important not to miss the opportunity for early treatment. Azathioprine and mesalazine can also cause pancreatic toxicity, so if serum pancreatic enzyme levels increase during the treatment of inflammatory bowel disease, drug-related pancreatitis must also be considered[15].

Infectious disease

Mumps is often encountered in daily clinical practice, but few patients develop pancreatitis that requires additional treatment. Pancreatitis as a complication is reported in 0.3%-15% of patients when mild cases are included[16]. Abdominal symptoms such as pain and tenderness may occur before the clinical onset of mumps (4-8 d after viral infection) and often spontaneously resolve in about 1 wk. In addition, pancreatitis may occur without parotid

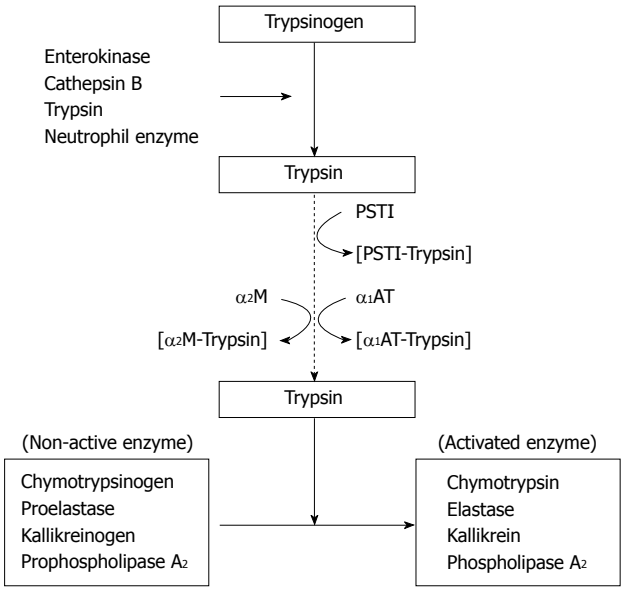

Figure 1 Suppression mechanisms for pancreatic enzyme activation. PSTI: Pancreatic secretory trypsin inhibitor; α2M: α2-macroglobulin; α1AT: α1-antitrypsin.

gland swelling in a few patients. When pancreatitis of unknown etiology occurs, testing for the mumps virus is recommended. Two deaths have been reported to date, so although rare, possible serious infection must be kept in mind[17].

Pancreatitis associated with mycoplasma infection is broadly classified into two types: early onset type during early infection (days 1-3) and late-onset type after respiratory tract symptoms have occurred (days 7-14). The mechanism in the former is thought to be direct invasion of mycoplasma into the pancreas, and in the latter, pancreatic injury caused by autoantibodies to acinar cells[18]. The prognosis in pancreatitis due to mycoplasma is generally good.

Congenital anomalies

Among anomalies of the pancreatobiliary system, choledochal cyst is the most common cause of acute pancreatitis[1,2,4,19]. In fact, many choledochal cysts are discovered because of symptoms of acute pancreatitis. In children with acute pancreatitis in whom the etiology is unclear, ultrasonography, endoscopic retrograde cholangiopancreatography (ERCP), or magnetic resonance cholangiopancreatography (MRCP) should be performed[20,21]. Most choledochal cysts, with the exception of Todani classification type Ⅱ (bile duct diverticulum) and type Ⅲ (choledochocele), are associated with abnormal union[22]. The sphincter of Oddi is usually most thickened in the duodenal muscularis mucosa; however, in abnormal union, because this sphincter surrounds a common channel after union of the main pancreatic duct and common bile duct, there is communication between the ducts during sphincter contraction[23]. Therefore, reflux of bile into the pancreatic duct, a protein plug in the common channel, or gallstone impaction is probably involved in the onset of pancreatitis.

PANCREATITIS CAUSED BY GENETIC MUTATIONS

Hereditary pancreatitis is due to autosomal dominant inheritance with about 80% penetrance. A relationship between a mutation in the cationic trypsinogen gene (protease serine 1, *PRSS1*) and hereditary pancreatitis was identified in 1996[24]. In 2000, a mutation in the serine protease inhibitor gene (*Kazal* type 1: *SPINK1*) was reported to be related to chronic idiopathic pancreatitis of unknown cause[25]. Patients with hereditary pancreatitis due to a *PRSS1* gene mutation or relapsing pancreatitis due to a *SPINK1* gene mutation can develop pancreatic exocrine insufficiency and diabetes in the future, and they are a high-risk group for pancreatic cancer[26-28]. The cause of these complications like cancer, as in chronic pancreatitis due to other etiologies, involves hyperplasia and metaplasia of the pancreatic duct epithelium due to recurrent or chronic inflammation. *K-ras* gene mutations also play a role[29]. Diabetes or pancreatic cancer developing in childhood cases has not been reported.

Recently, variants in *CPA1,* which encodes carboxypeptidase A1, were implicated in early onset pancreatitis in children up to 10 years old. The mechanism by which *CPA1* variants confer increased pancreatitis risk may involve misfolding-induced endoplasmic reticulum stress rather than elevated trypsin activity[30].

Other causes

In malignant lymphoma, lymphoma invasion near the head of the pancreas may compress the pancreatic duct and lead to acute pancreatitis[31]. In addition, in solid pseudopapillary neoplasms, intratumoral hemorrhage due to trauma can cause transient tumor enlargement, leading to pancreatic duct obstruction and acute pancreatitis[32].

PATHOPHYSIOLOGY

To understand the pathophysiology of acute pancreatitis, knowledge about the inhibitory mechanisms of activation of pancreatic enzymes under physiological conditions is necessary. In normal pancreatic acinar cells, lysosomes containing cathepsin B, which are involved in intracellular and extracellular digestion, and zymogen granules containing digestive proenzymes, such as trypsinogen, are released; and these inactive proenzymes remain inactivated[33,34]. In addition, even if trypsin is aberrantly activated in the pancreas for some reason, its activity is blocked by pancreatic secretory trypsin inhibitor (PSTI). Moreover, if trypsin leaks into the blood, the endogenous trypsin inhibitors α1-antitrypsin (α1AT) and α2-macroglobulin (α2M) bind to trypsin and suppress its activity (Figure 1)[35]. Anatomically, the sphincter of Oddi located in the duodenal ampulla of Vater prevents reflux of duodenal fluid into the pancreatic duct. Pancreatic duct pressure is also usually higher than bile duct pressure, so there is no bile reflux into the pancreatic duct[23].

Excessive stimulation of pancreatic exocrine secre-

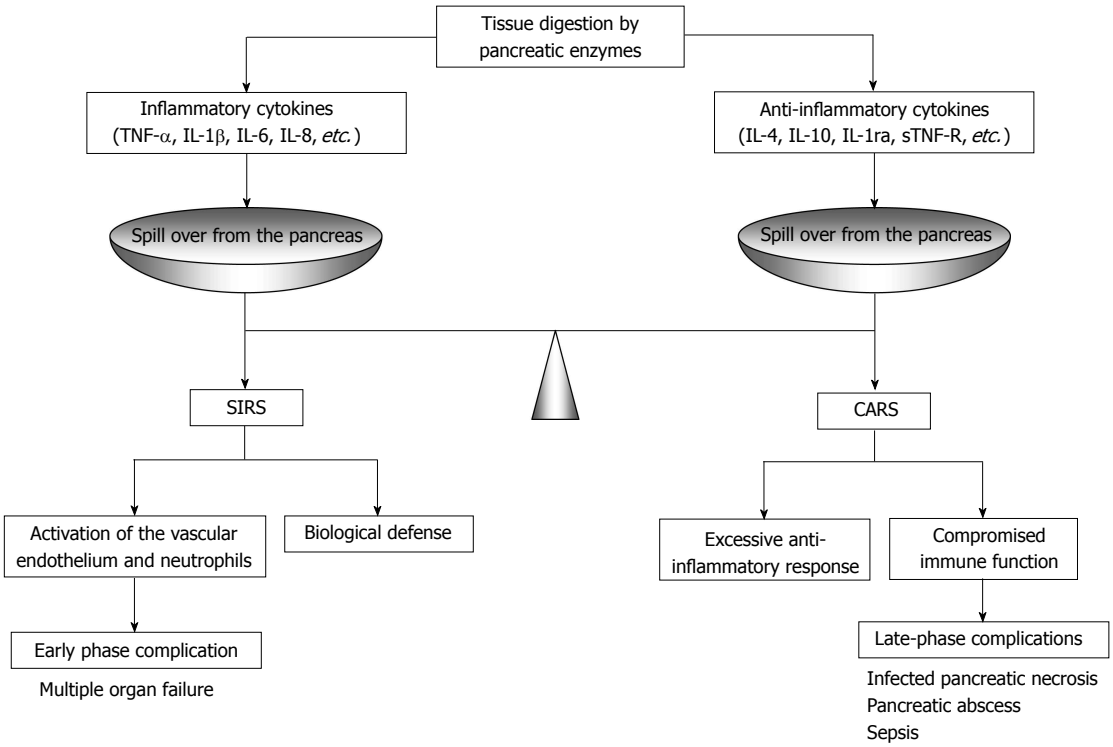

Figure 2 Compensatory anti-inflammatory response syndrome and systemic inflammatory response syndrome during acute pancreatitis. TNF: Tumor necrosis factor; IL: Interleukin; sTNF-R: Soluble tumor necrosis factor receptor; CARS: Compensatory anti-inflammatory response syndrome; SIRS: Systemic inflammatory response syndrome.

tions can cause reflux of pancreatic juices and enterokinase, pancreatic duct obstruction, and inflammation. These conditions can disrupt the above-mentioned defense mechanisms, activate trypsin beyond the ability for trypsin inactivation, and increase attacking factors, thus leading to acute pancreatitis[36]. Enterokinase is the most efficient activator, but trypsin itself, lysosomal enzymes (cathepsin B) in pancreatic acinar cells, and neutrophilic enzymes are also activators[34,36]. In experimental models of early acute pancreatitis, blockage of secretion has been suggested as the initiating event, leading to the accumulation of zymogen granules within acinar cells. This event is followed by a co-localization of digestive enzymes and lysosomal enzymes within vacuoles and, finally, an activation of enzymes that cause acute intracellular injury[37]. The activation of zymogen protease in pancreatic acinar cells is thought to play an important role in the development of acute pancreatitis[36,38].

Mild pancreatitis mainly involves the pancreas and local surrounding lesions. It is generally reversible, and about 6 mo after clinical remission, the pancreas recovers its normal morphology and function. In severe pancreatitis, vasoactive substances such as histamine and bradykinin are produced in large amounts with trypsin activation. As this vasoactive process increases, third spacing of fluids and shock due to hypovolemia may occur. In addition, leakage of activated enzymes from the pancreas causes secondary cytokine production. These cytokines trigger the systemic inflammatory response syndrome (SIRS)[39,40]. SIRS results in hyperactivation of macrophages and neutrophils throughout the body and the release of tissue injury mediators; multiorgan failure, including shock, circulatory failure, and acute respiratory distress syndrome (ARDS), may occur[41-43].

Meanwhile, as a biological defense response, anti-inflammatory cytokines and cytokine antagonists are induced to prevent prolongation of SIRS. This predominance of cytokine antagonists is called compensatory anti-inflammatory response syndrome (CARS)[44]. Because CARS inhibits new cytokine production, susceptibility to infection is increased, and infection of vital organs can occur. As a result of infection, endotoxins in the blood stimulate neutrophil aggregation in distal organs, tissue injury mediators are released, and distal organ failure occurs (Figure 2).

CLINICAL DIAGNOSIS AND ASSESSMENT OF SEVERITY

The diagnosis of acute pancreatitis is in principle based on clinical findings, biochemical tests, and imaging studies. Both a differential diagnosis and assessment of severity are necessary. The etiology of acute pancreatitis in children often differs from that in adults, and differences in the clinical manifestations and course may occur. Therefore, the diagnosis should be made keeping in mind specific features of the disease in children and after obtaining a past medical and family medical history (Figure 3).

Clinical manifestations

More than 90% of adults with acute pancreatitis report

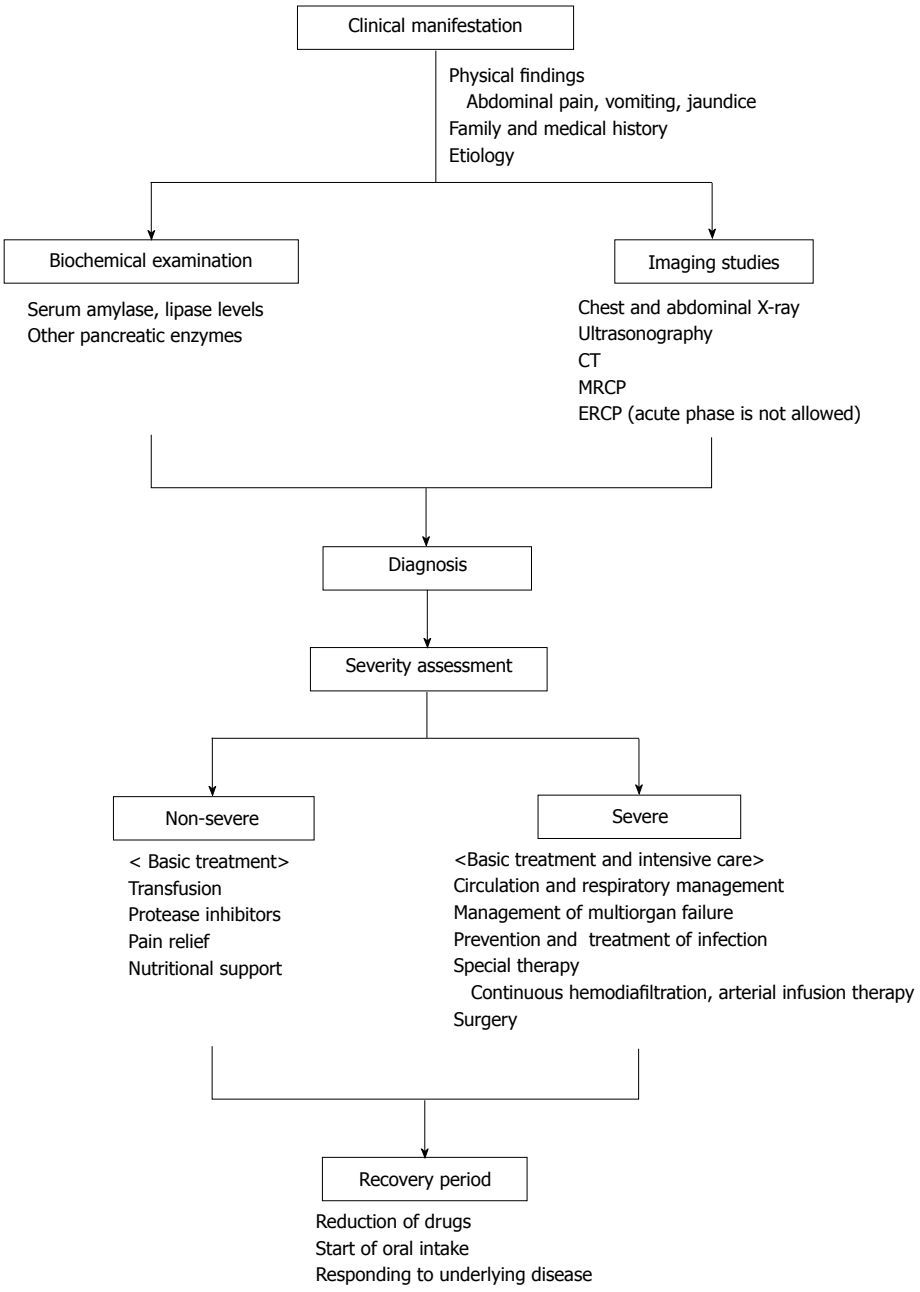

Figure 3 Clinical diagnosis of acute pancreatitis. CT: Computed tomography; ERCP: Endoscopic retrograde cholangiopancreatography; MRCP: Magnetic resonance cholangiopancreatography.

abdominal pain[45,46]. Abdominal pain is also an important early symptom in children. Weizman *et al*[47] reported that all 61 of their pediatric patients with acute pancreatitis initially had abdominal pain. Ziegler *et al*[48] also reported abdominal pain in 40 of 49 patients (82%). Table 3 shows the initial symptoms by age in our series of 135 children with acute pancreatitis[19]. In older children, the frequency of abdominal pain as a first symptom was similar to that in adults, whereas in younger children, vomiting was an important clinical symptom[49]. However, very young children and those with mild pancreatitis sometimes have non-specific abdominal pain. The location, characteristics, and triggers of abdominal pain, as well as physical examination of the abdomen, are important clues in the diagnosis of acute pancreatitis.

Other symptoms may include jaundice, fever, diarrhea, back pain, irritability, and lethargy. Jaundice and clay-colored stools suggest an abnormality of the biliary system such as a choledochal cyst, and there may be a palpable abdominal mass[8]. Infants and toddlers cannot verbalize abdominal pain, but vomiting, irritability, and lethargy are common[48]. In severe acute pancreatitis, children may initially present with shock, followed by symptoms of multiorgan failure, including dyspnea, oliguria, hemorrhage, and mental status changes[1].

Laboratory investigations

The prompt measurement of serum amylase is useful for

Table 3 First symptoms and chief complaints by age *n* (%)

	Age, yr			
	1-5 (*n* = 53)	6-10 (*n* = 47)	11-17 (*n* = 35)	Total (*n* = 135)
Abdominal pain	46 (86.8)	39 (83.0)	32 (91.4)	116 (85.9)
Fever	21 (39.6)	21 (44.7)	10 (28.6)	52 (38.5)
Vomiting	29 (54.7)	16 (34)	6 (17.1)	51 (37.8)
Jaundice	9 (17)	2 (4.3)	0	11 (8.1)
Back pain	0	1 (2.1)	5 (14.3)	6 (4.4)
Pale stool	3 (5.7)	1 (2.1)	0	4 (3)
Diarrhea	0	1 (2.1)	2 (5.7)	3 (2.2)
Loss of consciousness	1 (1.9)	1 (2.1)	1 (2.0)	3 (2.2)
Others	5 (9.5)	2 (4.2)	2 (5.8)	9 (6.6)

a diagnosis of acute pancreatitis[50]. However, elevated levels are also seen in gastrointestinal diseases such as pancreatobiliary tract obstruction and perforative peritonitis, as well as in salivary gland disease and renal failure. Therefore, low disease specificity is a problem. Serum lipase has a sensitivity of 86.5%-100% and specificity of 84.7%-99.0% for diagnosing acute pancreatitis[51]. Thus, its sensitivity is higher compared to serum amylase. In severe pancreatitis, serum lipase levels 7 times higher than normal have been reported within 24 h after onset of pancreatitis[52]. The degree of elevation and serial changes, however, generally do not correlate with disease severity[53]. In acute pancreatitis due to ASNase or valproic acid, which is fairly common in children, serum amylase may not be elevated[13]. Therefore, other serum pancreatic enzymes should also be measured.

Imaging

When acute pancreatitis is suspected, plain chest and abdominal X-rays are essential. A plain chest X-ray may show a pleural effusion, ARDS, or pneumonia. Although these findings are not specific for acute pancreatitis, they are important for the assessment of disease severity. A plain abdominal X-ray may show an ileus, colon cut-off sign, sentinel loop sign, calcified gallstones, pancreatic stones, or retroperitoneal gas. This information is important in assessing the clinical course of acute pancreatitis and is necessary for a differential diagnosis to rule out other diseases such as gastrointestinal perforation[54,55].

Ultrasonography is a convenient and non-invasive test. It is the test of first choice for screening to diagnose acute pancreatitis in children and for following the clinical course. The ultrasound diagnosis of acute pancreatitis is based on pancreatic morphology, appearance of the pancreatic parenchyma and pancreatic duct, and extrapancreatic findings[56,57].

CT scanning together with ultrasonography is essential for diagnosing acute pancreatitis. CT is useful to evaluate any extrapancreatic lesions, monitor the clinical course, and assess severity. In particular, CT is superior for early assessment of acute pancreatitis when ultrasound findings are nonspecific because of abdominal gas[56,58].

Pancreatitis in children is often caused by pancreatobiliary tract anomalies such as a choledochal cyst or abnormal union of the pancreatobiliary junction. Therefore, ERCP should be performed in pancreatitis of unknown cause. MRCP imaging has also improved and is useful in searching for a cause of acute pancreatitis in children[59]. In particular, MRCP should be performed before ERCP to detect any pancreatobiliary tract disease in children with initial onset of acute pancreatitis of unknown cause. However, in younger children, abnormal union of the pancreatobiliary junction is often difficult to delineate[21].

Severity assessment

Rapid and accurate assessment of severity is useful for selecting appropriate initial treatment and predicting the prognosis. In 2002, DeBanto *et al*[1] were the first to suggest a scoring system for predicting the severity of acute pancreatitis in children. This system is modified from the Ranson and Glasgow systems and consists of the following eight parameters: age (< 7 years old), weight (< 23 kg), white blood cell count at admission (> 18500 cells/μL), lactic dehydrogenase at admission (> 2000 U/L), 48-h trough Ca^{2+} (< 8.3 mg/dL), 48-h trough albumin (< 2.6 g/dL), 48-h fluid sequestration (> 75 mL/kg per 48 h), and 48-h rise in blood urea nitrogen (> 5 mg/dL). They set the cutoff for predicting a severe outcome at three criteria. However, this scoring system is not exact for Asian children[18]. Lautz *et al*[2] also reported that DeBanto pediatric scores have limited ability to predict acute pancreatitis severity in children and adolescents in the United States. Recently, we reported the usefulness of a new severity assessment that modified the acute pancreatitis severity scoring system of the Ministry of Health, Labour and Welfare of Japan (JPN score) for use in children[60,61]. The parameters of the pediatric JPN score were as follows: (1) base excess ≤ -3 mEq or shock (systolic blood pressure cutoffs according to age group); (2) PaO_2 ≤ 60 mmHg (room air) or respiratory failure; (3) blood urea nitrogen ≥ 40 mg/dL [or creatinine (Cr) ≥ 2.0 mg/dL] or oliguria (< 0.5 mL/kg per h); (4) lactate dehydrogenase ≥ 2 × the value of the upper limits; (5) platelet count ≤ $1 \times 10^5/mm^3$; (6) calcium ≤ 7.5 mg/dL; (7) C-reactive protein ≥ 15 mg/dL; (8) number of positive measures in pediatric SIRS score ≥ 3; and (9) age < 7 years old or/and weight < 23 kg. The cutoff for predicting a severe outcome was set at three criteria.

The CT severity index has proven to be very useful in adults[62]. Recently, Lautz *et al*[58] also reported that the CT severity index was superior to a clinical scoring system for identifying children with acute pancreatitis at heightened risk for developing serious complications.

TREATMENT

The initial treatment for acute pancreatitis is to withhold oral intake of food or fluid to allow the pancreas to rest (*i.e.*, prevent stimulation of pancreatic exocrine secretions). Fluid and electrolyte supplementation, enzyme inhibition therapy, and treatment to relieve pain and

prevent infection are provided. It is important to gradually permit liquid and food intake at a suitable time while continuing treatment. This treatment strategy is based on a consensus conference and evidence accumulated in adult patients. The basic pathogenesis of acute pancreatitis does not greatly differ between adults and children, and the treatment selected for children should be similar to that in adults.

Infusion of extracellular fluid

Because fluid leaks into the surrounding tissue due to inflammation associated with acute pancreatitis, adequate infusion to supplement extracellular fluid is needed during initial treatment. In severe cases, increased vascular permeability and decreased colloid osmotic pressure causes extravasation of extracellular fluids into the surrounding tissue and retroperitoneum and then into the peritoneal cavity and pleural cavity, thus leading to large losses in circulating plasma volume[63]. This acute circulatory impairment causes a rapidly deteriorating condition in early acute pancreatitis.

DRUG THERAPY

Analgesics

Pain in acute pancreatitis is often intense and persistent, and pain control is required. Appropriate use of analgesics can effectively reduce pain, but this should not interfere with making a diagnosis or providing other treatments[64-66]. The analgesics used include pentazocine, metamizole, and morphine.

Antibiotics

In mild cases of acute pancreatitis, the incidence of infectious complications and mortality rates are low, and prophylactic antibiotics are usually not necessary. However, even in mild cases, antibiotics should be considered if severity increases or complications like cholangitis develop. In severe cases, antibiotics can reduce infectious pancreatitis complications and improve the prognosis[67]. Drugs should be selected with good tissue distribution to the pancreas.

Pancreatic protease inhibitors and octreotide

The Santorini Consensus Conference in 1997 concluded that gabexate mesilate did not contribute to reduced mortality rates in acute pancreatitis[68]. However, in severe acute pancreatitis, continuous infusion of large doses of gabexate mesilate may decrease complications and mortality rates[69]. Similar efficacy in children has been reported, but no clear evidence exists[70]. Protease inhibitors may be a part of combined modality therapy (especially to improve hemodynamic status), but judicious administration is advised in severe cases.

Octreotide was introduced in the early 1980s and offers several advantages over somatostatin, such as a much longer half-life and the option for either subcutaneous or intravenous administration[71]. Octreotide is a powerful inhibitor of exocrine pancreatic secretion and cholecystokinin production[72]. Several studies have evaluated the effect of octreotide on the incidence of clinical pancreatitis after ERCP and postoperative complications such as pancreatic duct fistula following pancreaticoduodenectomy and pancreatic transplantation[73,74]. Effectiveness in reducing complications in acute pancreatitis has not been demonstrated[75]. However, at the case report level, octreotide has been effective in treating pancreatic pseudocysts as a complication in acute pancreatitis and in preventing and treating drug-related pancreatitis due to ASNase, a key drug used to treat lymphocytic leukemia in children[76-78]. As a somatostatin derivative, the most common adverse effect of octreotide is abdominal distention, but adverse effects such as failure to thrive are unlikely if octreotide is given for only 2-6 wk.

NUTRITIONAL SUPPORT

In severe pancreatitis, the early initiation of enteral nutrition reduces the incidence of infections and leads to shorter hospital stays[79]. An enteral feeding tube is placed in the duodenum or in the jejunum past the ligament of Treitz[80]. This type of nutrition is recommended to reduce stimulation of exocrine pancreatic secretion.

Control of abdominal pain and serum pancreatic enzyme levels should be considered in deciding when to resume oral intake. If serum pancreatic enzymes are decreasing, overall status is good, and abdominal pain has subsided, liquid intake can be started. If serum amylase and lipase levels are approximately less than two times the upper normal limits, a fat-restricted diet should be started[81]. Energy and fat intake can gradually be increased with careful monitoring.

Specific treatment for severe pancreatitis

In patients with infected pancreatic necrosis, surgical drainage and pancreatectomy may be indicated. Specific treatments such as continuous hemodiafiltration to remove humoral mediators and continuous regional arterial infusion of a protease inhibitor and antibiotics have been effective in adults[82,83]. These specific treatments have also been effective and lifesaving in children[84,85]. Although there is no universally acceptable scoring system for predicting the severity of childhood acute pancreatitis, consideration should be given to early transfer of severe patients to a medical center where intensive treatment is available.

Endoscopic treatment and surgery

Anatomic anomalies such as abnormal union of the pancreatobiliary junction are an indication for surgery. In patients with outflow tract obstruction of pancreatic juices caused by ampulla of Vater anomalies or pancreatic divisum, endoscopic sphincterotomy is effective.

Infectious complications should be clinically suspected if fever or signs of inflammation recur during the course of acute pancreatitis. Symptoms often become

prominent 2 wk or more after the onset of pancreatitis. The definitive diagnosis of infected pancreatic necrosis can be made by CT- or ultrasound-guided local fine-needle aspiration and bacteriologic cultures[86,87]. However, this procedure may be difficult in children. Therefore, worsening blood test results, positive blood cultures, positive blood endotoxins, elevated serum procalcitonin levels, and CT findings of the pancreas may serve as clues to a diagnosis of infected pancreatic necrosis[88].

Patients whose general condition is stable can be conservatively treated with antibiotics and observed, but if their condition does not improve, a necrosectomy is required. Necrosectomy early in pancreatitis is associated with a high mortality rate, so it should ideally be performed after the patient's hemodynamic status and general condition have stabilized[89]. Percutaneous necrosectomy, endoscopic transgastric necrosectomy and laparoscopic pancreatic necrosectomy have recently been reported as less invasive treatments in adults and a few children[90-92]. Pancreatic abscesses generally require percutaneous, endoscopic, or surgical drainage.

Pancreatic pseudocysts are cysts that develop due to injury of the pancreatic duct and extravasation of fluid. These occur 4 wk or later after the onset of pancreatitis. Treatment is indicated for pseudocysts if their size does not decrease, if they are accompanied by abdominal pain, or if there are complications of infection or hemorrhage. Endoscopic ultrasound-guided transgastric puncture and drainage can safely be performed in these cases[93,94].

CONCLUSION

Currently, our approach to acute pancreatitis in children mainly depends on physician experience and knowledge gained from acute pancreatitis in adults. Acute pancreatitis in children tends to be considered a difficult disease, even by pediatric gastroenterologists. However, with recent advances in diagnostic techniques and treatment methods, unfamiliar and difficult diseases are becoming controllable diseases once they are better understood. In order to improve treatment outcomes in patients with childhood acute pancreatitis, future studies focusing on developing a scoring system for predicting the severity of acute pancreatitis and identifying the potential effective treatment modalities for children should be conducted.

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TOPIC HIGHLIGHT

WJGP 5th Anniversary Special Issues (3): Pancreatitis

Genetics of acute and chronic pancreatitis: An update

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Abstract

Progress made in identifying the genetic susceptibility underlying acute and chronic pancreatitis has benefitted the clinicians in understanding the pathogenesis of the disease in a better way. The identification of mutations in cationic trypsinogen gene (*PRSS1* gene; functional gain mutations) and serine protease inhibitor kazal type 1 (*SPINK1* gene; functional loss mutations) and other potential susceptibility factors in genes that play an important role in the pancreatic secretory functions or response to inflammation during pancreatic injury has changed the current concepts and understanding of a complex multifactorial disease like pancreatitis. An individual's susceptibility to the disease is governed by genetic factors in combination with environmental factors. Candidate gene and genetic linkage studies have identified polymorphisms in cationic trypsinogen (*PRSS1*), *SPINK1*, cystic fibrosis trans-membrane conductance regulator (*CFTR*), Chymotrypsinogen C (*CTRC*), Cathepsin B (*CTSB*) and calcium sensing receptor (*CASR*). Individuals with polymorphisms in the mentioned genes and other as yet identified genes are at an enhanced risk for the disease. Recently, polymorphisms in genes other than those involved in "intra-pancreatic trypsin regulatory mechanism" namely Claudin-2 (*CLDN2*) and Carboxypeptidase A1 (*CPA1*) gene have also been identified for their association with pancreatitis. With ever growing number of studies trying to identify the genetic susceptibility in the form of single nucleotide polymorphisms, this review is an attempt to compile the available information on the topic.

Key words: Chronic pancreatitis; Acute pancreatitis; Genetic susceptibility; Single nucleotide polymorphisms; Inflammation

Core tip: Pancreatitis is a progressive inflammatory disease. Though the pancreas has adequate protection against environmental and metabolic stress, if the magnitude of this stress exceeds the threshold which the organ can handle, it leads to pathologic effects. Although genetic variables have been identified that affect the function of pancreas, namely polymorphisms in serine protease inhibitor kazal type 1 (*SPINK1*), polymorphisms in cationic trypsinogen (*PRSS1*) and Chymotrypsinogen C (*CTRC*) genes in the acinar cells and cystic fibrosis trans-membrane conductance regulator (*CFTR*), calcium sensing receptor (*CASR* genes) in the ductal cells leading to pancreatitis, off late many genetic factors outside of the "intra-pancreatic trypsin regulatory mechanism" have been identified for their role in pancreatitis. This review is an update on the genetic aspects of acute and chronic pancreatitis.

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INTRODUCTION

Chronic pancreatitis (CP) is a disease associated with

inflammation where the secretory parenchyma of the pancreas is progressively destroyed. There is involvement of several known risk factors and processes such as inflammation, necrosis, apoptosis or duct obstruction despite the heterogeneity in pathogenesis. The process of fibrosis usually leads to progressive worsening in lobular morphology, structure of pancreas, changes in arrangement and composition of the islets and deformation of the large ducts[1]. These conditions lead to diabetes that is due to irreversible morphological and structural changes and exocrine and endocrine dysfunction[2]. The major types of pancreatitis are acute pancreatitis (AP), recurrent acute pancreatitis (RAP) and CP.

In spite of an individual carrying a genetic risk and being subjected to oxidative or metabolic stress, the pancreas is histologically normal in appearance in the pre-acute phase. "First hit" in terms of injury due to excess alcohol consumption, metabolic factors, hyperlipidemia, gallstones and genetic factors leads to AP-which is a sentinel AP event (SAPE)[3]. During this proinflammatory phase, inflammatory related damage occurs due to the infiltration of the pancreas with inflammatory cells. This phase may end through an anti-inflammatory response that is mediated partly by tissue macrophages and is associated with the activation of stellate cells and subsequent proliferation causing fibrosis. However clinical recovery is attained in most of the cases.

If this phase is followed by RAP due to genetic risks namely polymorphisms in serine protease inhibitor kazal type 1 *(SPINK1),* polymorphisms in cationic trypsinogen (*PRSS1*), cystic fibrosis trans-membrane conductance regulator (*CFTR*) genes and other as yet unknown genes, or chronic cell stressors develop like alcohol, smoking, oxidative stress, *etc.*, after the SAPE (second hit), it leads to CP which is due to chronic inflammation and progressive fibrosis. CP may also manifest as a direct result of extensive pancreatic necrosis, duct obstruction in the proximal region directly resulting from severe AP which is independent and without the second hit[4].

Many risk factors that contribute varyingly to pancreatitis have been identified. These include alcohol, metabolic factors, toxins, insecticides, certain medications, viral and bacterial infections, trauma caused by surgery[5]. Growing evidence suggests a substantial contribution of genetic predisposition to pancreatitis. As early as 1950's, genetic studies on pancreatitis suggested that it may be an inherited disease[6]. After this initial description, a mutation inherited in autosomal dominant mode was identified in the cationic trypsinogen gene that is located on 7th chromosome in individuals with hereditary pancreatitis[7,8]. Further to this, a number of other mutations/polymorphisms in genes that have a role in inhibition, regulation or modulation of the pancreatic trypsin activity, secretory function and inflammatory injury respectively were identified. Mutations in the *PRSS1*, *SPINK1*, *CFTR* and polymorphisms in other genes namely the ones regulating the response to inflammation [tumor necrosis factor (TNF), interleukin-1 (IL-1) and IL-10][9] are the major genetic contributors to the development of AP and CP.

A model (two hit model) for the pathogenesis of pancreatitis has been proposed[10], suggesting that "there is a loss of balance between events associated with activation and degradation of active trypsin enzyme leading to the presence of persistent "super-trypsin" with in the acinar cell that is due to mutations or polymorphisms in genes namely *SPINK1,* Cathepsin B (*CTSB*), Chymotrypsinogen C (*CTRC*) and other yet to be identified susceptibility genes. This loss of balance leads to inflammation and these events are the first hits that contribute to the pathogenesis of pancreatitis". The presence of additional genetic and/or environmental risks leading to one or more phenotypes namely fibrosis, stone formation and/or diabetes and these events are the second hit.

AP: DEFINITION, SYMPTOMS AND RISK FACTORS

AP is a syndrome of acute and sudden inflammation of the pancreas. Clinically, it is detected by upper abdominal pain with sudden onset, digestive enzymes namely pancreatic amylase and lipase that are elevated in the serum and/or typical findings like edema, peripancreatic fat stranding, fluid collection on the abdominal imaging studies. The process in AP is initiated by an injury that is acute followed by an inflammatory response (also acute) which is mostly out of proportion and to the extent of tissue injury. The above response is due to premature activation of digestive enzymes in the pancreas that digest the tissue, consequently activating the inflammatory cascade. The immune system may also be cross-activated by the activated pancreatic digestive enzymes. Many risk factors for AP have been identified. The most important of them being duct obstruction by gall stones, parasites, tumors, anatomical abnormalities and endoscopic retrograde cholangio-pancreatography; metabolic factors like hyperlipidemia, hypercalcemia and acidosis; toxins like ethyl alcohol, insecticides, scorpion toxins, medications (azathioprine, NSAIDs, tetracycline, *etc.*); Bacterial and viral infections, trauma caused by blunt or penetrating or surgery apart from genetic susceptibility namely mutations in *PRSS1*, *SPINK1* and *CFTR*[5].

CP: DEFINITION, SYMPTOMS AND RISK FACTORS

CP is a disease associated with inflammation that is progressive and is characterized by three main features. Abdominal pain that is recurrent or persisting at the clinical level, damage of the parenchyma in pancreas with irregular sclerosis and inflammation, accompanied by ductal dilation, strictures or stones at the morphological level and finally a progressive loss of exocrine and endocrine functions at the functional level[11-13]. Based on the etiologies and risk factors, a working classification for CP

Table 1 General genetic information of the genes which confer susceptibility to pancreatitis[1]

Name of the gene	Chromosome	No. of splice variants	Length (bp) of exon region	No. of exons
CTRC	1	4	898	8
CASR	3	4	5009	7
PRSS1	7	6	800	5
CTSB	8	35	3875	10
SPINK1	5	3	542	4
CFTR	7	11	6128	27
CLDN2	X	3	3150	2

[1]Extracted from ENSEMBL. *CTRC*: Chymotrypsin C; *CASR*: Calcium sensing Receptor; *PRSS1*: Trypsinogen Gene; *CTSB*: Cathepsin B; *SPINK1*: Serine protease inhibitor kazal type 1; *CFTR*: Cystic fibrosis transmembrane conductance regulator; *CLDN2*: Claudin 2.

has been elaborated by the American Gastroenterological Association according to its prevalence and mechanism named TIGAR-O classification system (toxic-metabolic, idiopathic, genetic, autoimmune, recurrent and severe AP, obstruction)[14]. The toxic metabolic include alcohol, smoking (tobacco), hyperlipidemia, hypercalcemia, chronic renal failure and certain medications; idiopathic includes early onset, late onset and tropical; mutations in cationic *PRSS1* gene, *CFTR* gene, *SPINK1*, α-1 antitrypsin deficiency and other unidentified genes comprise genetic risk; autoimmune includes isolated autoimmune chronic pancreatitis, autoimmune syndromic CP including Sjogren's syndrome-associated CP, primary biliary cirrhosis-associated CP and inflammatory bowel disease-associated CP. Recurrent and severe AP-associated CP includes post necrotic (severe AP), vascular disease/ischemic and post-irradiation. Obstructible risk factors include sphincter of Oddi disorders, pancreas divisum, duct obstruction (tumor), preampullary duodenal wall cysts and post-traumatic pancreatic duct scars.

GENETIC RISK FACTORS FOR AP AND CP

It has long been suggested that inappropriate activation of trypsinogen in the pancreas is the first and most important step in the development of pancreatitis[15] and all the known genetic susceptibility factors for pancreatitis identified till date can be categorized as members of the intra-pancreatic trypsin regulatory mechanism and were identified employing a candidate-gene approach based on the above mechanism and they include polymorphisms/mutations in genes namely *CTRC*, *CASR*, Trypsinogen gene (*PRSS1, 2* and *3*), Cathepsin B (*CTSB*), *SPINK1/PST1*, *CFTR* gene. General information about the genes is presented in Table 1. A recent study[16] identified an underlying genetic susceptibility in approximately half of idiopathic CP patients, when they screened for mutations in *PRSS1, SPINK1, CTRC* and *CFTR* genes, emphasizing the important role of genetics in CP. A detailed list of different types of polymorphisms identified in these genes till date has been extracted from ENSEMBL and presented in Table 2 and the list of polymorphisms in these genes are also listed in the web site www.pacreasgenetics.org, however only the important polymorphisms/mutations have been discussed in detail in this review.

Trypsinogen (PRSS1, 2 and 3) genes

PRSS1 (cationic trypsinogen), anionic trypsinogen (*PRSS2*) and mesotrypsinogen (*PRSS3*) are the three types of trypsinogen that are expressed by the pancreas to an extent of two-thirds to one-third to less than 5% respectively[17,18]. Eight trypsinogen genes are shown to be located in the beta T-cell receptor locus at 7q35[19]. The *PRSS1* gene that is mapped to the long arm of chromosome 7 encodes the trypsin-1 (TRY-1) protein[8,20]. Important mutations (gain of function namely A16V, N29I, R122H) have been identified in the *PRSS1* gene that are associated with hereditary pancreatitis in Caucasians[21,22], French[23], D162D variant in Chinese[24] however a study from India reported that *PRSS1* gene mutations are not associated with CP[25]. A study from Korea reported that 5.4% of subjects with idiopathic CP and 40% with pancreatitis that is hereditary carried R122H mutation in the *PRSS1* gene and other variants were not reported apart from R122H. None of the 50 controls had the mutation[26]. One important study[27] screened for *PRSS1* mutations in a Belgian patient with sporadic CP and observed a migration pattern that is altered different from the transition (g.133283G > A) in exon 3 of the gene. Subsequent analysis by DNA sequencing revealed a DNA variant that was novel (g.133283-133284GC > AT) also resulting in R122H, however they concluded that in contrast to the change in codon CGC to CAC, codon CGC > CAT strongly suggested an alternative mutational mechanism of gene conversion.

Apart from the polymorphisms and their associations with pancreatitis, studies have also looked in to the copy number variations (CNVs) for their role in pancreatitis. A study[28] identified a duplication and triplication of 605kb segment on chromosome 7q35 in French ICP patients, which increased the copy number of *PRSS1* and 2 genes that code for cationic and anionic trypsinogen. The same study identified a trypsinogen gene that was hybrid with exon 1, 2 from *PRSS2* and exons 3 to 5 from *PRSS1*, which had two gain of function effects namely increase in trypsinogen gene copy number with N29I mutation in it. The 605kb segment duplication was also assessed further in French and Indian patients with idiopathic CP (ICP) and concluded that it was associated with French ICP but not in Indian patients with CP[29], however the CNVs in *PRSS3* were not associated[30].

Serine protease inhibitor Kazal type 1/pancreatic secretory trypsin inhibitor gene

SPINK 1/pancreatic secretory trypsin inhibitor (*PSTI*) is a specific trypsin inhibitor and an acute phase protein which is secreted by the acinar cells[31]. The gene encoding *SPINK1* has 4 exons and 3 introns that is located at

Table 2 Summary[1] of the polymorphisms in genes related to pancreatitis

Name of the gene	Upstream gene variants	Downstream gene variants	Non-coding exon variants	Synonymous variants	Missense variants	Stop gained	Intron variants
CTRC	490	430	102	28	57	5	789
CASR	580	732	129	433	1459	57	4707
PRSS1	1031	1634	431	126	280	6	637
CTSB	5763	11413	621	682	1261	10	18675
SPINK1	366	252	38	8	37	0	236
CFTR	1193	2377	87	447	2533	558	13723
CLDN2	205	171	0	36	78	0	560

[1]Extracted from ENSEMBL Upstream Gene variants: A sequence variant located 5′ of a gene. Downstream gene variants: A sequence variant located 3′ of a gene. Non-coding exon variants: A sequence variant that changes non-coding exon sequence. Synonymous variants: There is no change in the resulting aminoacid. Missense variants: Variant that changes one or more bases, resulting in a different aminoacid but where the length is preserved. Stop gained: Sequence variant whereby at least one base of a codon is changed, resulting in premature stop codon, leading to a shortened transcript. Intron variants: a variant occurring within an intron. *CTRC*: Chymotrypsin C; *CASR*: Calcium sensing Receptor; *PRSS1*: Trypsinogen Gene; *CTSB*: Cathepsin B; *SPINK1*: Serine protease inhibitor kazal type 1; *CFTR*: Cystic fibrosis transmembrane conductance regulator; *CLDN2*: Claudin 2.

5q32 and is approximately 7.5kb long[32]. SPINK1 protein plays a role in the prevention of premature activation of zymogen that is catalyzed by trypsin within the pancreatic duct system or the acinar tissue. A reactive site in the protein serves as a specific target substrate for trypsin[33] and it can inhibit up to 20% of the activity of pancreatic trypsin. It is the first line of defense against auto digestion, thereby protecting the pancreas[9], however inhibition of trypsin by *SPINK1* is temporary as trypsin may target the trypsin-SPINK1 complex and subsequently degrade the inhibitory molecule and restore trypsin activity[34]. *SPINK1* mutations cause a loss of function mutations as against *PRSS1* which generate gain of function mutations. There are several mutations/polymorphisms that are identified till date in the *SPINK1* gene (Table 2), however N34S is the most common missense mutation, that is a substitution of asparagine by serine at codon 34. N34S polymorphism was found in individuals especially without a family history and many studies have confirmed its association in different ethnic groups[25,35-37]. A substantial number of patients (15%-40%) with ICP carry N34S mutation in either heterozygous or homozygous state based on the above studies. The *SPINK1* polymorphisms (N34S) are in complete linkage disequilibrium with other variants that are located in the introns[38]. Other mutations/polymorphisms have also been identified namely a promoter mutation (-215-A and -215 G > T), a mutation in the start codon that destroys the only translational initiation codon of *SPINK1* (2 T-C, Met to Thr; MIT)[39], -53C > T; -41G > A, -2C > A; L14P; D50E; IVS3 + 125C > A; IVS3 + 184T > A; R65Q; R67C which were reported predominantly in single patients or families[35,38,40].

Polymorphisms in *SPINK1* gene are generally associated with loss of function. Although the *SPINK1* N34S polymorphism is associated with pancreatitis, the association is weak with very few individuals with the mutation developing pancreatitis some time during their life time[35,41]. Furthermore there is no difference in the severity of the disease with respect to the heterozygous and homozygous genotypes of SPINK1; there are complex interactions and the effect of the mutation depends on the reduction in the enzyme. Pancreatitis may be initiated in the homozygous N34S state, however the heterozygous genotype may only cause a lowering of the enzyme level and it requires other additional factors (genetic and environmental) to initiate the disease[42]. Therefore in general *SPINK1* polymorphism is hypothesized to be a susceptibility factor for a polygenic complex trait or a disease modifier[3] with polymorphisms in other genes being involved.

Apart from the above polymorphisms, two copy number mutations (deletions) in the SPINK1 gene that were associated with loss of function and encoding pancreatic secretory trypsin inhibitor (*PSTI*) were identified by a study[38]. In a particular family these deletions were co-inherited with a missense mutation (p.L997F) in the *CFTR* gene, suggesting complex interactions between the CNVs and single nucleotide substitutions contributing to the disease phenotype. SPINK1 polymorphisms are common in the general population (approximately 2%) but are shown to be significantly associated with pancreatitis.

Chymotrypsin C gene

CTRC encodes Chymotrypsin C, a digestive enzyme. It is produced by the acinar cells in the pancreas. It is packaged with zymogen granules and is secreted along with other digestive enzymes from the pancreas. Prematurely activated trypsin is destroyed by CTRC by acting on the molecule within the calcium-binding loop in the absence of calcium and therefore is a crucial candidate gene in the pathogenesis of pancreatitis[43]. Many polymorphisms have been identified in this gene till date (Table 2). A study[44] had sequenced all the 8 exons (8.2 kb) of the *CTRC* gene in a total of 621 individuals with idiopathic or hereditary CP and 614 control subjects of German origin and identified that the large majority of the variants were in 2nd, 3rd and 7th exons. Only exons 2, 3 and 7 were sequenced in an additional 280 CP patients and 2075 controls for exons 2 and 3 and 2190 controls for exons 7. Although a number of missense and deletion variants were found they concluded that the two most frequent variants

which were significantly overrepresented in the pancreatitis group as compared to the controls were c.760C > T (p.R254W) and c.738_761del24 (p.K247_R254del) (30/901 (3.3%) affected individuals but only in 21/2804 (0.7%) controls), both of which were located in exon 7. Furthermore, this group also studied 71 and 84 individuals of Indian origin with tropical pancreatitis and controls respectively, and suggested a higher frequency of *CTRC* alterations in this cohort [10/71 (14.1%) in Tropical pancreatitis Vs 1/84 (1.2%) controls] as compared to the German cohort and two relatively frequent variants were found in the Indian cohort namely c.217G > A (p.A73T) missense alteration and the c.190_193del ATTG (p.I64LfsX69) frame shift deletion[44]. Another study from India[45] identified 14 variants in 584 CP patients and 598 normal subjects [71/584 CP patients (12.2%) and 22/598 controls (3.7%)], when all the eight exons and flanking regions of the *CTRC* gene were sequenced. It was p.V235I variant which was common in the Indian CP patients as against the p.K247_R254del variant in the Caucasians. Apart from this variant the study also identified other pathogenic variants namely p.A73T and c.180C > T as significantly associated with Indian CP.

Cathepsin B gene

The human *CTSB* is 25.6kb. It has 12 exons. Several transcript species are known to be produced by alternative splicing[46]. It is hypothesized that chronic pancreatitis is a result of mutations in the *CTSB* gene and they may be involved in premature activation of trypsinogen or inappropriate localization[47]. A study on the CTSB gene polymorphisms and tropic calcific pancreatitis identified significant association of Val26Val polymorphism (allele frequency of 0.48 in patients *vs* 0.30 in controls) with Odds of 2.15 apart from differences in the mutant allele frequencies that are significant at Ser53Gly (allele frequency of 0.10 *vs* 0.04 in patients and controls respectively) and C595T SNPs (allele frequency of 0.12 *vs* 0.20 in patients and controls respectively. Further L26V polymorphism was equally as common in N34S positive and wild type patients suggesting that CTSB is involved independently with the disease. This study suggested that *CTSB* polymorphisms may be associated with pancreatitis more so in the absence of mutations in *PRSS1* gene and N34S *SPINK1* polymorphism proposed to play a disease modifier role[47], however another study failed to associate polymorphisms in this gene with pancreatitis in European cohort (allele frequency of 0.398 in patients and 0.48 in controls)[48].

Calcium-sensing receptor gene

Auto-activation and autolysis are processes in which trypsinogen molecule is activated to trypsin and is also degraded by other trypsin molecules. For the mentioned purpose, two specific cleavage sites exist for potential attack by other trypsin molecules. Lysine 23 (L23) is the first site and arginine 122 (R122) the second. The cleavage of L23 causes trypsinogen activation to trypsin with 8-amino acid trypsinogen activation peptide being released while R122 cleavage causes inactivation of trypsin. The susceptibility of the two sites for an attack is regulated by calcium concentration and concentration dependent occupation of the calcium binding sites[49]. In normal acinar cells low calcium concentrations are prevalent and these low concentrations limit the activation of trypsinogen, thereby promoting inactivation of trypsin by exposing the second site (R122), however calcium hyper stimulation or dysregulation in the acinar cells favors activation of trypsinogen and prevention of trypsin inactivation[50]. Thus regulation of calcium levels (intra-acinar) is critical for preventing trypsinogen activation and pancreatic injury. CASR plays a major and important role in maintaining the calcium homeostasis through its effect on renal tubules and parathyroid gland. A variety of hypercalcemia-associated syndromes are associated with genetic variants in the *CASR* gene[51]. The first of the reports associating *CASR* mutations with CP came from a family study of 5 individuals who were all heterozygous for the N34S *SPINK1* polymorphism. Only two of the 5 heterozygous individuals developed CP and both these individuals presented with a T > C mutation at position 518 in the *CASR* gene, that is a leucine to proline amino acid change in the extracellular domain of the CASR protein[52], suggesting that *CASR* mutations may be a predisposing genetic factor that may increase the susceptibility for CP. Another study[53] that screened for mutations in *SPINK1* and *CASR* gene on a small Indian cohort of 35 patients with Tropical chronic pancreatitis (TCP) and an equal number of controls reported that a combination of mutations in both the genes was seen in 6% of the patients, while 22% had mutation in single gene, suggesting that *CASR* mutations may be a risk for TCP and that risk may be further increased with associated *SPINK1* mutation. A study by Muddana *et al*[54] initially included 115 subjects with pancreatitis and 66 controls. Of the study group, 57 patients and 21 controls were predetermined to carry the N34S *SPINK1* polymorphism. Based on the initial results, the study included an additional 223 patients and 239 controls to analyze the three common non-synonymous SNPs in exon 7 that were found to be significant from the initial study. The *CASR* exon 7 polymorphism (R990G) was significantly (Odds, 2.01 and $P = 0.01$) associated with CP and the association of this SNP was stronger in subjects with moderate to heavy alcohol consumption. This study however did not find any significant associations between the various *CASR* genotypes and *SPINK1* N34S in CP. None of the earlier reported polymorphisms from Germany and India were also detected in this US-based study. All the association studies suggest that recurrent trypsin activation/dysregulated calcium and failed inhibition increase the risk of pancreatitis *via* the intracellular calcium dysregulation.

CFTR gene

The impact of *CFTR* gene continues to be debated, although variants in this gene are strongly associated

with pancreatitis. *CFTR* gene in humans has 27 exons, is located at 7q31 and is 250 kb in length[55]. For the proper functioning of the duct cells in the pancreas and other anion secreting epithelial cells, CFTR anion channel is a critical molecule. CFTR apart from regulating the functions of other channels also conducts both chloride and bicarbonate channels, the opening and closing of which controls the bulk of fluid secretion from the pancreas[50]. The association between idiopathic CP and *CFTR* mutations was demonstrated in 1998[56,57]. More than 1200 mutations have been identified and based on the mechanism by which they disrupt the function; they are classified in to five different groups with group V mutations subsequently being included in group I (as they cause functional alterations in the levels of mRNA)[58]. Class I mutations affects biosynthesis, class Ⅱ mutations affect protein maturation, class Ⅲ affect chloride channel regulation/gating while class Ⅳ mutations affect chloride conductance[59]. An additional class of mutations was proposed by Haardt *et al*[60] as class Ⅵ which included protein stability mutations.

A higher frequency of mutations in the *CFTR* gene was seen in a significant number of patients (30%) with ICP. There was six and two times higher frequency of CFTR mutations and 5T allele respectively in patients[56,57,61]. With few of these mutations there was a reduction in the amount of functional CFTR. The others might be a combination of a severe and a mild mutation or either type of mutations with 5T allele in intron 8 of the gene[9]. There is an increased risk (up to 40 fold) for pancreatitis when individuals are compound heterozygotes[62]. Complete coding sequences of the *CFTR,* P*RSS1* and *SPINK1* genes were analyzed for mutations and it was seen that 25%-30% of the patients with CP carried at least a single mutation in the *CFTR* gene and majority were compound heterozygotes for a *CFTR* mutation or were trans-heterozygotes for *CFTR, PRSS1* and *SPINK1* mutations[62,63]. Furthermore, a combination of two *CFTR* mutations and N34S in *SPINK1* gene increases the risk of pancreatitis by 900 fold[9]. It is clear from these studies that *CFTR* variants are associated with CP, however the mechanisms of the complex interactions of various susceptibility loci has to be understood in a better way.

Proinflammatory cytokine genes

It is already established that the cytokine profile with in the pancreas is different in CP as compared to normal pancreas[64]. A potential factor that could affect the production of proinflammatory cytokines are polymorphisms in these genes. Association studies involving polymorphisms in various cytokine genes have shown varying results in various populations. Various genes namely *TNF-a* (tumor necrosis factor-α), *Monocyte chemoattractant protein-1*, and *IL-8*[65-67] have been studied for their association with pancreatitis.

It is known that *TNF-a* along with *IL-1* is a major early cytokine to mediate the systemic inflammatory response syndrome (SIRS)[68-70]. A study[71] reported the association between *TNF-a* -238 AG but not -308 SNP genotype with organ failure (shock and/or respiratory failure) and in the IL-6 gene the CC genotype at position 174 was associated with biliary etiology of AP. The study included 84 patients with AP (no controls were included) and known polymorphisms in *TNF-a*, interleukin 1 (*IL-1*), IL-1 receptor antagonist (*IL1RN, IL-6* and *IL-10)* were genotyped for etiology associated susceptibility and severity, however other polymorphisms like *TNF-a*-1031, -863 and -857 SNPs were not included in the study. Another study[72] reported a negative association between *TNF-a*-308 and severity of pancreatitis (397 patients and 300 controls with major allele frequency in *TNF* gene being 0.87 for patients with AP and 0.86 for controls) from Finland, however they did not study the *TNF-a*-238 SNP. These results were similar to studies reported from United Kingdom, by[73], who studied 190 and 102 AP patients and controls respectively and Sargen *et al*[74], who studied 135 AP and 107 controls respectively (78.3% and 84.4% for *TNF-a*-308 and 21.7% and 15.6% for *TNF-a*-238 in controls and AP respectively). However, *TNF-a*-308 allele was reported to be associated with severe AP in Hungarian patients[75]. The study included 77 patients (mixed etiology and grouped according to the severity of the disease on the basis of Ranson scores) and 71 controls. Another study[76] associated *TNF-a*-308 allele with shock in patients with severe AP, however suggested that the polymorphism played no part in disease severity or susceptibility. The study included 208 AP cases and 116 ethnicity matched controls. A recent meta-analysis[77] integrated the previous findings on *TNF-a*-308 G > A and -238 G > A alleles and explored whether the polymorphisms were associated with susceptibility and severity to pancreatitis. The study included 1569 pancreatitis cases and 1330 controls from 12 published case-control studies and concluded that polymorphisms in these two sites did not alter the risk of pancreatitis.

Monocyte chemoattractant protein 1 (MCP-1) is a member of the C-C chemokine family. It exerts a strong chemo attractant activity in macrophages, lymphocytes and monocytes[78]. A common polymorphism-2518 A > G alters the expression of the gene with G allele being associated with higher levels of MCP-1 protein which is associated with higher risk of pancreatitis. A study from United States[65] included 77 consecutive patients and 116 controls for the mentioned genotype and concluded that the -2518 genotype is a risk factor for severe AP (12 of 14; 86% with AP *vs* 50 of 116; 43% control subjects) and also suggested that MCP-1 serum levels appear to be an accurate predictor of severity of AP and death when measured early in the course of the disease. Another study from Italy[79] studied 118 AP, 64 ARP, and 142 CP patients and 88 controls and concluded that all patients with pancreatic inflammatory disease had significantly higher serum MCP-1 levels. A study[80] which looked at the relationship between a polymorphism in the *MCP-1* gene (-2518A/G) and AP in the Han population of Suzhou, China suggested an increased risk of AP associ-

ated with G allele [72.4% (113/156) and 76.1% (35/46) in severe AP; 47.1% (113/240)]. However, the 2518A/G polymorphism in the *MCP-1* gene did not significantly alter the susceptibility to CP[81].

Interleukins are proinflammatory cytokines and polymorphisms in these genes have been shown to affect the immune response[82]. A meta-analysis[83] on the interleukin gene polymorphisms which included a total of 10 studies, covering a total of 1220 AP cases and 1351 controls showed evidence for significant association between *IL-8* -251 T/A (rs4073) polymorphism and AP risk, suggesting that *IL-8* -251 A allele was associated with an increased risk of AP. However, there were no significant associations between *IL-1* [IL-1 +3954 C/T (rs1143634) and IL-1 -511 C/T (rs16944)], IL-6 [IL-6 -174 G/C (rs1800795) and *IL-6* -634 C/G (rs1800796)] and *IL-10* [IL-10 -1082 A/G (rs1800896), *IL-10* -819 C/T (rs1800871) and *IL-10* -592 C/A (rs1800872)] gene polymorphisms and AP risk. In summary, the study concluded that the IL-8 -251 T/A polymorphism was associated with an increased risk of AP. In addition, there were no significant associations between IL-1, IL-6 and IL-10 gene polymorphisms and AP risk.

Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine. It is released by macrophages and lymphocytes[84]. It plays an important pathogenic role in AP and a study[85] investigated the role of -173 G > C polymorphism and the (CATT) n repeat microsatellite at position -794 in 164 patients with AP and 197 controls C allele 58/160 [18.1% in AP *vs* 47/097 (11.9%) in controls]. There was no significant difference in the repeat length of the microsatellite marker between patients and controls, however the C allele of the -173 G > C genotype was significantly higher in patients.

Claudin-2 and Carboxypeptidase A1 gene

New susceptibility loci for CP have been identified. The first SNP in the Claudin-2 (*CLDN2*) locus is the outcome of the first and only reported Genome wide association study for pancreatitis done till date, which included 1676 cases and 4507 controls in stage I and 910 cases and 4170 controls in stage Ⅱ. The study identified two SNPs namely one SNP in *PRSS1-PRSS2* locus (allele frequency of 0.576 in controls *vs* 0.634 in pancreatitis) and the other in the Claudin-2 locus (*CLDN2*) (allele frequency of 0.261 in controls *vs* 0.322 in pancreatitis). The SNP in the *PRSS1* locus affects susceptibility by altering the expression of trypsinogen and the SNP in the *CLDN2* is associated with atypical localization of claudin-2 in pancreatic acinar cells. Homozygous or hemizygous genotype (in females and males) confers the greatest risk and the alleles also interact with alcohol consumption to increase the risk of pancreatitis[86]. Another study[87] analyzed variants in Carboxypeptidase A1 (*CPA1*) encoding carboxypeptidase A1, primarily in Germany discover set and in thee replication sets from Europe, India and Japan. *CPA1* variants were associated with non-alcoholic CP with varying levels of significance in the discovery [29/944 (3.1%) of German cases and 5/3,938 (0.1%) controls] as well as all the three replication sets 8/600 (1.3%) of European cases and 9/2,432 (0.4%) controls, 5/230 (2.2%) of Indian cases and 0/264 controls and 5/247 (2.0%) of Japanese cases and 0/341 controls. The study concluded that variants may confer an increased risk of CP and the mechanism may involve endoplasmic reticulum stress that may be induced by misfolding rather than trypsin activity that is elevated.

GENETIC TESTING FOR *PRSS1, SPINK1* AND *CFTR* GENES - WHEN TO ORDER THE TEST?

A valuable diagnostic genetic test to investigate AP and CP has been added ever since a point mutation in the *PRSS1* gene has been identified. Consensus guidelines for ethical molecular genetic testing for hereditary pancreatitis has been proposed[88] which recommends it under the following conditions: (1) Unexplained two or more (recurrent) episodes of documented pain that are separate with hyperamylasemia attack; (2) Idiopathic CP; (3) Family history of pancreatitis [in a parent, sib, child (first degree) and in aunt, uncle or grand parent (second degree)]; (4) A need to exclude significant concern of hereditary pancreatitis in a child with an unexplained episode of documented pancreatitis that required a hospitalization; (5) As part of research protocol that is approved. Genetic testing (*PRSS1* mutations) in children below 16 years is indicated after; (6) Hospitalization that was required in an individual because of an episode of documented pancreatitis of unknown etiology that is severe enough; (7) Pancreatitis of unknown etiology in an individual with two or more documented episodes; (8) A child with an episode of documented pancreatitis, who has a relative with hereditary pancreatitis mutation that is known; (9) Recurrent abdominal pain (unknown etiology) in a child, where there is a distinct clinical possibility of hereditary pancreatitis; and (10) Diagnosis of hereditary pancreatitis as a distinct clinical possibility in an individual with CP of unknown etiology[88].

Currently genetic testing for mutations in SPINK1 or CFTR genes is considered as premature as the identification of mutations in these genes neither convincingly explains the disease in an individual who has been diagnosed with pancreatitis or has the ability to predict the possibility of developing the disease[88-90].

The significance of a positive test result for *PRSS1* genetic testing should be explained clearly to the subjects. Variable clinical course, mode of inheritance and incomplete penetrance are the important aspects apart from others, where counseling needs to be imparted to the patients. Strategies should be discussed to prevent future episodes of AP namely avoiding concomitant risk factors like alcohol, metabolic disturbances and drugs.

Important risk factors namely choledocolithiasis and other obstructive factors that contribute to AP have to be

identified and treated. Therefore patients have to be advised to undergo radiological and endoscopic evaluation to identify the above risks[91]. Furthermore, as these mutations (R122H or N29I) also significantly increase the risk for pancreatic cancer, the patients should be counseled for abstinence from tobacco and smoking[92] and counseling may be imparted and genetic testing ordered for at risk relatives if warranted[3].

CONCLUSION

As emphasized earlier many of the susceptibility loci identified till date have taken the candidate-gene approach and to the best of our knowledge there are no GWAS (Genome wide association studies) which are available apart from the only study which identified *PRSS1* and *CLDN2* polymorphisms recently[86]. Furthermore, a better understanding of the interactions of the etiological factors with susceptibility SNPs will aid in diagnosing and treating the disease at an early stage. There is an urgent need to utilize the advances in genomics namely GWAS and/or exome sequencing on NGS platform to unravel as yet unidentified susceptibility loci for pancreatitis, which is a multifactorial and a complex disease for a better understanding at the molecular level.

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TOPIC HIGHLIGHT

WJGP 5th Anniversary Special Issues (4): Barrett's

Molecular markers and imaging tools to identify malignant potential in Barrett's esophagus

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Abstract

Due to its rapidly rising incidence and high mortality, esophageal adenocarcinoma is a major public health concern, particularly in Western countries. The steps involved in the progression from its predisposing condition, gastroesophageal reflux disease, to its premalignant disorder, Barrett's esophagus, and to cancer, are incompletely understood. Current screening and surveillance methods are limited by the lack of population-wide utility, incomplete sampling of standard biopsies, and subjectivity of evaluation. Advances in endoscopic ablation have raised the hope of effective therapy for eradication of high-risk Barrett's lesions, but improvements are needed in determining when to apply this treatment and how to follow patients clinically. Researchers have evaluated numerous potential molecular biomarkers with the goal of detecting dysplasia, with varying degrees of success. The combination of biomarker panels with epidemiologic risk factors to yield clinical risk scoring systems is promising. New approaches to sample tissue may also be combined with these biomarkers for less invasive screening and surveillance. The development of novel endoscopic imaging tools in recent years has the potential to markedly improve detection of small foci of dysplasia *in vivo*. Current and future efforts will aim to determine the combination of markers and imaging modalities that will most effectively improve the rate of early detection of high-risk lesions in Barrett's esophagus.

Key words: Barrett's esophagus; Esophageal adenocarcinoma; Gastroesophageal reflux disease; Dysplasia; Biomarkers; Endoscopic imaging

Core tip: This review highlights recent advances and future directions in biomarker development and endoscopic imaging technology for identification of patients at risk of malignant progression of Barrett's esophagus.

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INTRODUCTION

Esophageal adenocarcinoma (EAC) has increased in incidence in the United States and other Western countries by at least six-fold in the past three decades, making it the cancer with the most rapid rise in incidence[1]. Prognosis is dismal at the time of diagnosis, with a five-year survival rate that remains below 20%[2]. This is particularly sobering in light of the longstanding recognition of Barrett's esophagus as a premalignant condition and of the technological advancements allowing for improved early detection and intervention.

Barrett's esophagus (BE) is defined as a replacement of normal squamous epithelium in the esophagus with columnar mucosa (endoscopic diagnosis), which is confirmed by biopsy as intestinal metaplasia (histologic diagnosis). Debate persists regarding the histologic requirement (such as presence of goblet cells) as well as the lack of distinction between short and long segment BE[3]. It is the leading risk factor for EAC, conferring a relative risk of 30-60 compared with that of the general population[4]. The pathophysiology of Barrett's metaplasia is incompletely understood but is related to chronic damage from gastric acid and bile reflux[5]. Strong association has been demonstrated between chronic gastroesophageal reflux disease (GERD) and both BE and EAC[6-8], but the nature of the progression from GERD to BE to EAC is less clear[9].

While BE is found in 5%-10% of patients with chronic GERD, most patients do not progress to EAC[10]. Moreover, most EAC are diagnosed incidentally, without a known history of GERD or BE[11], and quite often in advanced stages less amenable to cure. Thus from a public health standpoint, the key questions are: which members of the general population should be screened for BE, which patients with BE are likely to progress to EAC, and what surveillance program is appropriate[12]. In this review, we discuss the current understanding of Barrett's progression, recent advances in biomarker and endoscopic imaging development, and implications for future research and clinical practice.

EPIDEMIOLOGICAL MARKERS

In addition to chronic GERD, several risk factors for BE are well-established, including age over 50 years, male sex, white race, obesity, intra-abdominal fat distribution, and presence of hiatal hernia. Screening endoscopy may be appropriate for patients meeting several of these criteria[3,13]. Unfortunately, the vast majority of patients diagnosed with EAC have no prior diagnosis of BE, and many patients diagnosed with BE have no prior GERD symptoms[9].

CURRENT SURVEILLANCE PRACTICES

Current United States society guidelines recommend endoscopic surveillance of patients with documented BE[3,9,13] for the presence of EAC or its precursor lesions low-grade or high-grade dysplasia (LGD or HGD, respectively). This consists of regularly scheduled white light endoscopy with four-quadrant biopsies taken at 2 cm intervals, or 1 cm in patients with known or suspected dysplasia (Seattle protocol)[13]. Even when applied rigorously, this approach samples only a small fraction of the mucosal surface, and retrospective evidence suggests that in practice, the number of biopsies taken is often considerably lower than recommended, creating further sampling error[14]. This is especially problematic since early dysplastic lesions typically occur as small foci and can readily evade detection by the standard endoscopic biopsy practice regimen. Furthermore, these biopsy samples are typically not fully sectioned and examined; instead only a few sections from each sample are reviewed, which represents yet another order or two of magnitude decrease in actual tissue examined[15]. The present definitions of LGD and HGD are based on morphologic distinctions as graded by a pathologist; although interobserver reproducibility has been shown to be high at the ends of the spectrum (BE *vs* HGD or EAC), there appears to be considerable variation in separating nondysplastic BE from LGD or indeterminate dysplasia[12,16]. This non-concordance is even greater in the community setting, where a recent study demonstrated marked over-diagnosis of LGD following review of samples by a panel of expert pathologists[17].

These distinctions are important in practice because they have bearing on the likelihood of progression to EAC and consequently the need for close surveillance or intervention. For example, in the aforementioned study, patients with a consensus histologic diagnosis of LGD went on to develop HGD or EAC at a rate of 13% per year, whereas those downgraded to nondysplastic BE (NDBE) progressed at a rate of only 0.49% per year[17], although other studies suggest a lower incidence of LGD to HGD/EAC progression[18,19]. This is in keeping with data from recent large multicenter studies and meta-analyses, which estimate a low overall rate of progression from NDBE to EAC, on the order of 0.12%-0.38% per year, with very low mortality from EAC[19-21]. These findings, coupled with a lack of strong evidence showing mortality benefit, have led some health economists to argue that routine endoscopic surveillance of all patients with BE is likely not cost-effective[22], although at present it remains supported by guidelines[3,13].

ADVANCES IN THERAPY

Recent years have also seen the development and evaluation of endoscopic ablative techniques for dysplastic BE, which hold the promise of cancer prevention analogous to the current practice of polyp resection in the colon. Endoscopic mucosal resection has proven to be an effective therapeutic intervention in many patients with HGD or even intramucosal carcinoma and is associated with lower morbidity than surgical resection, although risk of cancer recurrence is higher in patients with lesions not strictly confined to the mucosa[23,24]. Radiofrequency ablation (RFA) has been shown to have high efficacy in the eradication of dysplasia and intestinal metaplasia as well as a good safety profile[25], and this effect appears to be durable[26]. In light of these encouraging findings and the high mortality of EAC, some experts have reintroduced the question of whether all BE should be ablated[27,28]. At present, while it appears to be cost-effective to ablate all HGD, it is less clear whether ablation of all LGD or NDBE is reasonable public health policy[29]. In addition, such efforts are complicated

by the presence of subsquamous intestinal metaplasia (SSIM), or "buried Barrett's," which can persist after ablation, is difficult to detect using current practice methods, and whose significance as a premalignant condition is as yet undetermined[30].

NEED FOR NEW BIOMARKERS

In this context, the main unresolved issue in BE management is to improve identification of those patients at highest risk for developing EAC.

The term "biomarker" broadly encompasses physiologic measurements, molecular analyses, or endoscopic or imaging findings[31]. The National Cancer Institute has established an Early Detection Research Network, which has developed a recommended biomarker validation pipeline encompassing a discovery phase, translational phase, and clinical implementation phase[32]. An ideal biomarker would objectively detect all dysplastic BE without significant false-positive results leading to unnecessary testing and intervention. As discoveries of such markers are few and far between, it is more realistic to expect that some combination of less-perfect markers will ultimately prove useful for the risk stratification of patients with BE. Many of the recent advances in biomarker research can be grouped into the categories of molecular markers and endoscopic imaging tools.

MOLECULAR MARKERS FOR DYSPLASTIC PROGRESSION

Much effort has been devoted in recent years to the search for a molecular marker that can serve as an adjunct to endoscopic and histologic surveillance in predicting malignant potential in BE. A recent comprehensive review of investigated and published molecular markers classifies them along the GERD-BE-EAC axis according to their potential usage as either diagnostic tools, indicators of progression, predictors of response to therapy, or aids in prognosis[33]. Of course, some markers span several of these denominations. Most of the hundreds of markers being evaluated are not yet approaching clinical utility, and another recent review article, using the same categories, discusses what requirements remain for clinical implementation of several of the more promising markers, such as larger prospective studies and external validation[31]. Since many of the molecular markers under investigation involve the differential expression of genes from normal to BE to dysplasia to EAC, another way to categorize these approaches could be where they fall along the axis of DNA to RNA to protein. Again, in some cases the same marker may be detected at multiple points along this axis.

Genetic coding

A hereditary component to BE and EAC has long been postulated[34] with reports of familial clustering, but most evidence has favored environmental rather than genetic risk factors[4]. A recent genome-wide association study, using large population-based epidemiological databases, compared patients with EAC to those with BE and normal controls. The authors report extensive polygenic overlap between BE and EAC and interpret this as evidence that the genetic basis for EAC is already present at the development of BE rather than occurring during progression. They identify several loci having strong association with both conditions, namely 19p13 in the oncogene-associated transcription coactivator gene *CRTC1*, 9q22 in esophageal speciation transcription factor gene *BARX1*, 3p14 near esophageal development transcription factor gene *FOXP1*, and 16p24 near the putative tumor suppressor gene *FOXF1*[35]. Further investigation will be needed to examine the clinical utility of genomic investigation as a screening or surveillance tool.

DNA content abnormalities are common among malignancies and preneoplastic states and involve all chromosomes. Several studies have demonstrated that such abnormalities, including aneuploidy, tetraploidy, and loss of heterozygosity at 17p and 9p loci, which affect the tumor suppressors p53 and p16, respectively, occur in EAC and may precede progression to cancer by up to 10 years[6,36]. Impressively, patients with all of these abnormalities in the setting of BE were found in one cohort to have a relative risk of EAC progression of 38.7 compared to patients with BE and none of the DNA abnormalities[36].

Epigenetics: DNA methylation

The role of epigenetics, defined as cellular information other than the DNA sequence itself that is heritable during cell division, in cancer development has been the subject of a growing body of literature since the 1980s[37]. An important epigenetic alteration is DNA methylation, which occurs almost exclusively at CpG nucleotides, found in high numbers in promoter regions, and is involved in the regulation of gene expression and silencing[37,38]. In malignancies, this may involve hypermethylation and consequent transcriptional repression of tumor suppressor genes or hypomethylation and increased expression of oncogenes[39].

Several recent studies have examined the role of DNA methylation in BE and EAC development. A genome-wide profiling, using microarray and hierarchical clustering analysis, of CpG methylation in esophageal tissue samples found that there was substantial difference in methylation pattern between normal esophagus samples and those with BE or EAC, but that the difference between BE and EAC was less clear[38]. This finding also suggests that the epigenetic, as well as genetic, alterations present in EAC may already be present in BE, thus suggesting potential markers for BE surveillance. However, a significant limitation of this study was that all of the BE samples were obtained from patients who developed EAC, as opposed to the vast majority of cases of BE that do not progress[38]. This weakness would become strength if future work demonstrates differences in methylation

patterns of these pre-malignant BE samples from those of nonprogressing, nondysplastic BE.

This was addressed by another recent study, which used DNA methylation arrays to differentiate between BE and EAC in tissue samples. This work delineated four genes (*SLC22A18*, *PIGR*, *GJA2*, and *RIN2*) which, when taken together, had an excellent receiver operating characteristic curve (AUC = 0.988) to distinguish BE from EAC. The authors applied this 4-gene methylation panel to a prospective multicenter study and presented evidence that it can detect nearby dysplasia or early neoplasia in endoscopic biopsies of BE even in the absence of visible histologic change in that particular sample, suggesting a field effect as observed in other types of malignancy. They proposed that patients with BE can be stratified into low, medium, and high risk of malignant progression using this panel as an adjunct to histopathologic evaluation but cautioned that follow-up data on its predictive power is not yet available[40].

Other publications focus on differential methylation of individual genes. As an example, endoglin, or ENG, is a transmembrane glycoprotein with a role in angiogenesis; hypermethylation of its encoding gene's promoter region has been associated with several cancers. Recently, this hypermethylation was found in human esophageal tissue, with frequency of 11.9% in normal esophagus and increasing sequentially to 13.3% in BE, 25% in dysplastic BE, and 26.9% in EAC. However, the frequency of ENG hypermethylation is greater in esophageal squamous cell carcinoma and thus may be more useful as a biomarker for this malignancy[41].

Epigenetics: microRNA

Another active field of research in cancer epigenetic markers is the use of microRNA (miRNA) signatures. MiRNAs are small, non-coding RNAs that regulate RNA translation including that of oncogenes and tumor suppressors; the current state of this research in esophageal cancer has recently been reviewed[42]. Based on analysis of multiple recent studies on miRNA in EAC and BE, the reviewers found that four miRNAs (miR-25, -99a, -133a, and -133b) have potential as diagnostic markers and five (miR-21, -27b, -126, -143, and -145) may have utility as both diagnostic and prognostic markers[42].

Two studies not included in the aforementioned review due to their very recent publication sought to assess the miRNA signature of BE and EAC using microarray analyses and hierarchical clustering, much like the DNA methylation studies described above and with similar results. A genome-wide analysis of miRNA expression levels showed clustering of BE and EAC signatures together as compared with that of normal esophageal tissue but interspersing of BE and EAC signals[43]. However, another study using microarray analysis showed a distinct pattern in EAC, with different patterns of up- and down-regulation seen in EAC compared with BE. This study also showed two miRNAs which were up-regulated in BE tissue adjacent to HGD lesions, again suggestive of a field effect for dysplasia that may be clinically useful alongside histologic surveillance[44].

Protein markers

The vast majority of molecular biomarker research in EAC has focused on differential expression of proteins in esophageal tissue. There are several recent review articles describing the state of this research, including a comprehensive list[33] and additional analysis[31], among others. Several promising and recently investigated classes of markers are described here.

One of the best-described cancer-associated proteins is the tumor suppressor p53. In a recently published large prospective case-control study, aberrant p53 expression by immunohistochemistry of biopsy samples was found to have a higher predictive value for neoplastic progression in BE than histologic diagnosis of LGD with strong inter-observer agreement among scoring pathologists. This association was seen with p53 overexpression, but even more strongly with loss of normal p53 expression[45]. This adds further support to prior studies using p53, including a case-control study which showed that using a combination of aneuploidy and overexpression of transcription factor Ki67 and p53 was predictive of neoplastic progression to HGD or EAC, independent of histology[46]. Another well-known protein in the cancer literature is human epidermal growth factor-2 (HER2), a proto-oncogene notorious for its role in predicting clinically aggressive breast cancers. A recent study using immunohistochemical and fluorescent in-situ hybridization methods on samples from patients with EAC showed a correlation between HER2 expression and p53 overexpression as well as early lesion protrusion[47].

Caudal homeobox transcription factor-2 (Cdx-2) is an intestine-specific transcription factor, but is expressed in BE, activated by acid and bile according to *in vitro* studies. It appears to help direct the development of intestinal metaplasia in BE[5]. Recent histologic and epigenetic research suggests that the encoding gene's promoter region is hypermethylated in HGD and intramucosal EAC; Cdx2 expression was correspondingly downregulated in dysplasia compared with BE metaplasia but restored in poorly differentiated invasive cancer, demonstrating gene silencing memory[48].

Stem cell markers have also received considerable attention as predictors of dysplasia and neoplasia in BE, in light of a newer theory of BE development and progression involving the activation of pluripotent esophageal stem cells to develop intestinal metaplasia in response to gastric acid and bile[5]. Leucine-rich repeat-containing G protein-coupled receptor 5 (Lgr5), a downstream target of the Wnt pathway and an intestinal stem cell marker, has been identified in immunohistochemical analyses of BE and shown to have increased expression in HGD and EAC as well as an apparent association with poor survival[49]. Doublecortin and CaM kinase-like-1, also a putative gastrointestinal stem cell marker, similarly has shown a progressive increase in expression from BE to dysplasia

to EAC by immunohistochemistry[50].

Cell signaling to control such processes as proliferation and apoptosis is tightly regulated by receptor tyrosine kinases (RTKs). Disruption of this balance is a common factor in various types of cancer[51]. A recent report showed increased expression and gene copy numbers of tyrosine kinase EPHB4 in both squamous cancer and adenocarcinoma of the esophagus, with corresponding supporting evidence in mouse and cell culture models[52]. Among the RTKs felt to be most promising as markers in EAC are EGFR, ErbB2, ErbB3, FGFR2, and Met, which have been shown to be up-regulated at early stages in dysplasia[53]. However, they have thus far met with mixed results as predictors of malignant progression, perhaps in part due to their heterogeneous expression among individual cancers[54]. A recent study described this heterogeneity using an RTK array; these differentially expressed proteins have great promise in therapeutics as targets of individualized therapy using different tyrosine kinase inhibitors depending on the RTK expressed *in vitro*[54]. For example, antibodies to EGFR and HER2 are promising therapeutic treatments for EACs expressing these particular RTKs[55-58]. The MAPK pathway, downstream of these individually varied RTKs, was frequently activated in pre-malignant and malignant states in human gene expression, representing another potential target for surveillance and treatment[54].

Another class of proteins known to have involvement in malignancy is that of mucins; these secreted and transmembrane glycoproteins function in limiting the activation of inflammatory responses and may become deregulated in states of chronic inflammation, leading to impaired epithelial repair and malignant transformation[59]. Based on initial immunohistochemistry analysis, regulation of different mucin proteins may be involved in BE progression, with decreased expression of the mucin aG1cNAc observed in Barrett's epithelium adjacent to EAC compared with nondysplastic BE when controlled for expression of the scaffold protein MUC6[60].

Literature on the use of NSAIDs including aspirin as therapy for BE has also evolved, and has included the use of prostaglandin E2 (PGE2) as a surrogate endpoint marker. PGE2 is associated with up-regulation of proliferation, resistance to apoptosis, angiogenesis, and increased cellular invasiveness and thus has a theoretically sound basis and utility in these research studies. However, it will need further validation for use as a clinical biomarker[61].

Molecular marker panels and associated conditions

Given the limitations and early phase trials of each of the above and many other candidate molecular markers when assessed alone, it is appealing to consider combining them as a panel and using with other associated risk factors to achieve better predictability of dysplastic progression. For example, given the known association between obesity and EAC has been shown[6], it stands to reason that markers of obesity may be predictive of malignant transformation. Indeed, in patients enrolled in the Seattle BE study (all with BE), increased levels of leptin and insulin resistance were associated with increased EAC risk, while increased high-molecular-weight adiponectin was inversely correlated with EAC[62].

A recent analysis of data from a nested case-control study assessed the utility of a panel of several established biomarkers (abnormal DNA content, p53, and cyclin A expression) and newer biomarkers (levels of sialyl Lewis-a, Lewis-x, and Aspergillus oryzae lectin (AOL) and binding of wheat germ agglutinin) on tissue samples from patients diagnosed with BE who either progressed or did not progress to EAC (cases and controls). A conditional logistic regression analysis was employed, which identified the best panel for risk prediction, consisting of LGD, abnormal DNA ploidy, and AOL. This panel of biomarkers conferred an odds ratio of 3.7 for EAC progression[63].

IMPROVING SAMPLING: NON-ENDOSCOPIC METHODS

A major limitation of the current molecular markers discussed above is that, no matter how sensitive or specific they may be in detecting dysplasia, they depend on adequate tissue sampling by random biopsies. Given the limitations of current endoscopic sampling practices as discussed above, a major remaining challenge is to improve the yield of tissue sampling. One approach relies on "field effect" of malignancies. This refers to the concept that genetic and environmental factors create a broad field of injury, upon which further insult leads to the formation of focal neoplasia[64]. As discussed above, some markers were present not only in areas of dysplasia or neoplasia but also in adjacent tissue, and the majority of genetic and epigenetic abnormalities were found to be already present in pre-dysplastic BE, illustrating this concept. A recent study investigated whether brushings from proximal squamous epithelium in patients with distal EAC exhibited intracellular nanoarchitectural changes as measured by partial wave spectroscopic microscopy, a technology that measures intracellular spatial distribution. Significant differences were observed using this technique, which is encouraging as it could allow for detection of distant malignancy with a minimally invasive approach[65]. However, by the time EAC is present it is often too late to intervene effectively, and it is presently unknown if a similar approach would detect earlier phases of dysplasia.

Several non-endoscopic techniques for screening and surveillance have garnered attention in recent years. One that has shown promise as a potential screening tool in the primary care setting is the Cytosponge. This is a sample acquisition technique in which a pill is swallowed following which a sponge expands in the stomach and is withdrawn *via* the esophagus, brushing off cells in the process. This is safe and well tolerated by patients in initial studies and has diagnostic potential when combined with a potential BE biomarker trefoil factor 3[66]. A microsimulation model predicts that screening 50-year-old

men with GERD using this technology would be cost-effective and reduce mortality[67]. A higher-tech approach to screening and perhaps surveillance is tethered capsule endomicroscopy, in which a pill-sized optical coherence tomography (OCT, see below) probe is swallowed and has the capability to obtain microstructure level imaging of the entire esophagus without requiring sedation[68].

SERUM BIOMARKERS

Although these less-invasive techniques show promise for reducing sampling error and achieving a broader screening population, they do not have the ease of use of a simple blood test. Researchers are working to find a biomarker that is present in the serum that could objectively aid in assessing risk of malignant transformation. Though such a marker has thus far proven elusive, several groups have demonstrated promising findings using antibodies to the well-described tumor protein p53. These antibodies form in response to overexpression of mutant p53 protein in patients with a variety of malignancies and are rare in serum from healthy control patients[69]. A study of serum samples of patients under endoscopic surveillance found a small number of patients who had detectable anti-p53 antibodies in serum samples taken before they were diagnosed with cancer[70]. A meta-analysis of 15 studies found that patients with esophageal cancer were approximately 7 times more likely to have serum p53 antibodies than those without cancer, but the marker was limited by poor and variable sensitivity[71]. A recent case report describes the post-operative surveillance of a patient with EAC over four years, showing lower titers of anti-p53 antibody in the serum after resection and suggesting utility of this marker to detect residual cancer in such patients[72]. These findings support the use of anti-p53 antibodies as a potential surveillance tool in patients with known BE or EAC, but its utility as a screening test in a broader population is not yet clear.

Panels including several biomarkers in combination may prove superior to individual markers alone in screening serum samples. Recently, use of serum biomarker panels was evaluated as a potential screening tool for the presence of BE in a VA population[73]. The best panel in this study included serum levels of several cytokines (IL 12p70, IL6, IL8, IL10), leptin, GERD frequency and duration, age, sex, race, waist-to-hip ratio, and *H. pylori* status. These were combined to give a biomarker risk score, with the highest equal to a 10-fold increase in risk of BE[73].

ENDOSCOPIC IMAGING TECHNIQUES

The mainstay of screening and surveillance of BE is standard white light endoscopy. Particularly with the increased resolution and high-definition monitors in current use, endoscopy is a successful screening modality as it allows for excellent visualization and the ability to sample tissue[6,74]. Dysplasia detection has been shown to increase with longer inspection time in patients with BE[75], a finding with clear relevance to the use of endoscopy as a surveillance tool. However, dependence on endoscopic surveillance with four-quadrant biopsies has to date not been successful in decreasing mortality from EAC and has raised concerns of cost-effectiveness, as mentioned above. Thus, a number of enhancements to conventional endoscopy are being explored to achieve more effective surveillance. An ideal imaging tool would improve objectivity, have a wide area of surveillance, produce results rapidly in real time, and have improved sensitivity and specificity for the detection of dysplasia compared to white light endoscopy. Current modalities in practice and under investigation were recently reviewed[74] and are discussed here.

Chromoendoscopy

The oldest and most "low-tech" of the available endoscopic image enhancements, chromoendoscopy involves the application of stains to mucosal surfaces during endoscopy to enhance visualization of musical surfaces. These stains are characterized as absorptive (*e.g.,* Lugol's iodine, methylene blue, toluidine blue), reactive (Congo read, phenol red), and contrast (indigo carmine)[76]. Methylene blue has been well studied in BE due to its propensity to stain intestinal metaplasia consistent with BE while sparing gastric mucosa, which may be useful for diagnosing short segment BE[74,77]. Widespread use of chromoendoscopy has been limited by variability of staining, laborious effort, and unclear correlation with dysplasia, and there is evidence demonstrating a lack of interobserver agreement or yield identifying early neoplasia in BE with the addition of indigo carmine or acetic acid to white light images[78]. More recent advances in endoscopic imaging have allowed for combination of chromoendoscopy with optical magnification, which has led to descriptions of characteristic relief patterns known as pit patterns[79,80]. While these patterns have shown to have good sensitivity for BE detection, a recent study found them to have low specificity, which may limit their clinical utility in targeting biopsies[81].

Optical enhancements

Improvement in digital endoscope technology has made endoscopic image enhancement possible without the mess of chromoendoscopy, earning the term "virtual chromoendoscopy." Narrow band imaging (NBI, Olympus) uses specific wavelengths of light to construct an enhanced image, and flexible spectral imaging color enhancement (Fujinon) and i-Scan EPKi processor (Pentax) apply digital filters to white light images[74]. NBI has been evaluated in BE. In the same study mentioned above for chromoendoscopy, NBI similarly failed to improve diagnostic yield or interobserver agreement[78]. On the other hand, a recent study demonstrates comparable or improved rates of BE detection but with fewer biopsies compared with standard methods[82], and a meta-analysis demonstrates high accuracy and precision in diagnos-

ing HGD in BE[83]. Thus this modality appears to have potential utility as both a screening and surveillance tool. Taken together, a meta-analysis and systematic review concluded that advanced imaging techniques using chromoendoscopy or virtual chromoendoscopy were found to improve diagnostic yield for dysplasia or cancer in patients with BE compared to white light endoscopy, but there was no significant difference in yield of detection between the two advanced imaging techniques[84].

Autofluorescence and trimodal imaging

Autofluorescence imaging takes advantage of endogenous fluorophores (*e.g.,* collagen, nicotinamide, adenine dinucleotide, flavin, and porphyrins), which can be stimulated by excitation (short-wavelength) light[85]. This has the advantage over white light endoscopy of producing real-time fluorescent images that may aid in detection, but initial systems have been limited by false positives from ulcers and inflammation rather than true dysplasia[85]. More recent efforts have combined autofluorescence with magnification endoscopy and narrow-band imaging ("trimodal imaging"), providing improved visualization of microvascular and microstructural architecture in malignant and premalignant gastrointestinal lesions[86]. Endoscopic trimodal imaging has been shown to be more effective in improving the targeted detection of HGD or EAC in BE[87].

However, this advantage seemed to no longer be present when trimodal imaging was evaluated in a community setting[88].

Fluorescent lectins

A more sophisticated adaptation of chromoendoscopy involves the targeted binding of markers, which are specific to areas of dysplasia. A recent study utilized the alteration in cell-surface glycans over the progression from BE to EAC. A fluorescently-tagged lectin, wheat germ agglutinin, was sprayed over the esophageal mucosa during endoscopy and was found to have specific binding permitting visualization of high-grade dysplastic lesions that were not visible by white light endoscopy alone[89]. This type of molecular imaging has considerable promise as a surveillance tool if findings are borne out in clinical trials.

Confocal laser endomicroscopy

A number of high-tech, high-resolution imaging modalities are currently under investigation. One of these is confocal laser endomicroscopy (CLE), which is in effect an endoscopic light microscope, enabling "optical biopsy" or near-histologic level of detail and tissue enhancement *via* the application of topical or IV contrast agents[74]. Existing commercial CLE systems are endoscope-based (Optiscan, Pentax) or probe-based (Cellvizio)[74]. A multicenter randomized-control trial using probe-based CLE showed significantly improved detection of neoplasia (HGD or EAC) compared with white light endoscopy[90]. Despite high specificity, there has been some concern about sensitivity of this method, which may be related to its limited field of view[91]. Early dysplastic changes are still being characterized, including pit patterns and possible vascular changes, but these remain largely subjective in interpretation. While this technology is promising and may have a role in specialized cases, application of this expensive, time-consuming, and operator-dependent modality in the community is unlikely to occur in the near future.

Optical coherence tomography

Another promising high-tech modality is optical coherence tomography (OCT), which is a high-resolution, cross-sectional imaging technique that utilizes backscattered light waves in a manner analogous to ultrasound with sound waves[92]. It has shown promising accuracy for detection of dysplasia and may help target biopsies[93]. OCT has several advantages as a surveillance tool – it has a wider field of view than confocal microscopy but similar resolution, does not require contrast administration, allows rapid image acquisition and 3-dimensional reconstruction, and can detect subsurface changes. This latter characteristic, the ability to visualize subsurface structures at greater depth than other modalities, enables accurate assessment of BE thickness and presence of SSIM before or after ablation, which in turn correlate with ability to achieve eradication of intestinal metaplasia using RFA[30,94,95]. Like other such modalities, though, OCT is presently costly and operator-dependent and likely has more of a future in tertiary centers. Given less distal optical requirements compared to confocal microendoscopy, however, OCT can be miniaturized for potential non-endoscopic screening of BE, as recently employed using a swallowed tethered capsule[68,96].

Elastic scatter spectroscopy

Elastic scatter spectroscopy (ESS) is related to optical scattering efficiency caused by optical index gradients of cellular and subcellular structures, allowing for detailed evaluation of microstructural features such as nuclear size, crowding and chromaticity, chromatin granularity, and mitochondrial and organellar size and density[97]. This technique has shown promise in preliminary studies, notably decreasing the number of biopsies required to diagnose dysplasia compared to the Seattle protocol[98], but more prospective data is needed.

Angle-resolved low-coherence interferometry

Another novel endoscopic imaging tool is angle-resolved low-coherence interferometry (a/LCI), which uses the distribution of elastically scattered light to make depth-resolved measurements of the size and index of refraction of cell nuclei. In BE, this can be employed to evaluate dysplasia up to significant depth, and preliminary studies indicate that it is accurate in doing so[99,100].

Raman spectroscopy

Finally, a tool that is being developed at present for en-

doscopic use is Raman spectroscopy (ERS), which relies on inelastic light scattering and can assess the biochemical components of its target, notably specific molecular constituents and signals. A recently published study reports high sensitivity and specificity of HGD and EAC detection and the ability to grade dysplasia, as well as the potential to combine ERS with narrow-band imaging for clinical application[101].

FUTURE DIRECTIONS: TOWARD A TARGETED AND OBJECTIVE APPROACH

Recent years have seen considerable research efforts devoted to the development of molecular markers and endoscopic imaging techniques to improve detection rates and diagnostic accuracy for esophageal adenocarcinoma and its premalignant conditions, BE and especially dysplastic BE. A great many molecular markers have been studied and are at varied phases of biomarker development using benchmarks established by the National Cancer Institute. Thus far, no single marker alone has shown sufficient improvement in accuracy of early detection compared with current guideline-based practice to warrant widespread clinical use. Perhaps the greatest promise has been shown by panels of several markers taken along with clinical risk factors and current endoscopic surveillance practices, which can be combined to yield risk scores similar to those used as predictive models in other disease states. Future biomarker research will likely focus on improving the predictive accuracy of these models.

A significant limitation to the ability to reliably detect small early foci of dysplasia on a background of metaplasia is the current reliance on random and limited, rather than targeted, sampling. Even a molecular marker with perfect sensitivity and specificity is only as good as the sample on which it is tested. Thus a major unmet need for improving detection will require improved endoscopic imaging modalities, likely used in combination, to locate such foci of dysplasia. This can be accomplished by improving visualization of the entire mucosal surface, using techniques such as microscopy, chromoendoscopy, optical enhancements, and fluorescence, or by using novel tools like CLE, OCT, ESS, a/LCI, or ERS to obtain an "optical biopsy" of subsurface structure and microstructure. Improvement in surface imaging may require combining imaging techniques, as has been illustrated by trimodal imaging, and further developments will likely validate and improve upon these methods. Subsurface imaging efforts will further confirm the correlations between optical findings and microstructural and biochemical composition. Optimal imaging tools will have the ability to evaluate broad areas of the esophagus, quickly hone in on those areas of highest significance, and have less dependence on subjective analysis when guided by simultaneously applied appropriate biomarkers.

Another way to mitigate the problem of sampling error is to take advantage of the field effect in malignant progression. This principle is relevant both for molecular marker and endoscopic imaging research. The prospect of using non-endoscopic sampling such as sponge or brush methods is appealing as a screening tool, if it can be combined with a sufficiently accurate marker. If field effect can be adequately demonstrated with a given marker on brush or biopsy samples, random sampling would be less troublesome for diagnostic purposes. Advanced optical imaging techniques have been investigated to detect ultrastructural cellular and vascular alterations suggestive of field effect in the colon cancer literature[64], and such efforts will likely be undertaken in the esophagus as well.

Even the most advanced endoscopic imaging techniques suffer from dependence on subjective interpretation by the endoscopist during examination, much as standard histologic evaluation of biopsy samples relies upon subjective determinations by the pathologist. Limiting this subjectivity in histopathology is a key goal of molecular marker development, and similar efforts should also be made in endoscopic imaging. Taking advantage of properties like autofluorescence and specific targeting of molecules to dysplastic foci *in vivo*, it may be possible to combine advanced imaging with molecular markers to achieve this goal. An ideal system would seamlessly integrate a marker of high predictive value with imaging technology allowing for microscopic level imaging of surface and subsurface structure, allowing for objective and targeted diagnosis and therapy.

As systems emerge that reliably demonstrate superiority to conventional approaches in the early detection of dysplasia and EAC, the degree to which they can be reasonably implemented as population-wide surveillance tools will become an important focus of investigation. These techniques require highly trained operators and at present are expensive and not widely available. At the outset, it can be expected that advanced modalities will be effective tools primarily at large academic centers, which may shift the responsibility of BE surveillance toward these institutions. As more providers become trained in the use of these systems and their cost decreases, their use in community settings should become more widespread.

CONCLUSION

Current screening and surveillance methods for the early detection of esophageal adenocarcinoma remain suboptimal given this cancer's increasing incidence and high mortality. Significant challenges include limitations in tissue sampling, lack of objectivity in describing premalignant states, and difficulties in targeting diagnostic and therapeutic modalities. Advances in biomarker development, from genetic and epigenetic characteristics to protein expression profiles, new approaches to sample acquisition, and novel endoscopic imaging tools allowing for improved surface and subsurface visualization, have shown considerable promise in addressing these issues. Future research endeavors will determine which

combination of markers and imaging techniques are most effective in detecting and decreasing mortality from esophageal adenocarcinoma.

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TOPIC HIGHLIGHT

WJGP 5th Anniversary Special Issues (4): Barrett's

Biomarkers of Barrett's esophagus

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Abstract

Barrett's esophagus is the strongest risk for esophageal adenocarcinoma (EAC). Metaplasia in patients with BE may progress to dysplasia and then invasive carcinoma. Well-defined diagnostic, progressive, predictive, and prognostic biomarkers are needed to identify the presence of the disease, estimate the risk of malignant transformation, and predict the therapeutic outcome and survival of EAC patients. There are many predictive and prognostic markers that lack substantial validation, and do not allow stratification of patients with gastroesophageal reflux disease in clinical practice for outcome and effectiveness of therapy. In this short review we summarize the current knowledge regarding possible biomarkers, focusing on the pathophysiologic mechanisms to improve prognostic and therapeutic approaches.

Key words: Barrett's esophagus; Esophageal adenocarcinoma; Biomarkers

Core tip: The importance of biomarkers of Barrett's esophagus is to provide identification of the disease, estimate the risk of malignant transformation, predict the response to therapy, and indicate the overall survival-prognosis for esophageal adenocarcinoma patients. Proposed predictive and prognostic markers do not allow stratification of gastroesophageal reflux disease patients for progression, outcome, and effectiveness of therapy in clinical practice. The aim of this short review is to discuss the current knowledge regarding proposed biomarkers to improve prognostic and predictive therapeutic approaches, with a focus on the pathophysiologic mechanisms.

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INTRODUCTION

Barrett's esophagus (BE) is characterized by the replacement of squamous epithelium in the esophagus by metaplastic columnar epithelium with goblet cells[1]. BE is a well-known risk factor for esophageal adenocarcinoma (EAC), a malignancy with the most rapid increase in incidence (approximately 500%) over the past 3 decades in the Western world, and with persistently poor outcomes when diagnosed after the onset of symptoms (survival less than 20% at 5 years)[2]. An important problem in treating the patients with BE is the absence of satisfactory surveillance programs in spite of the known stages of carcinogenesis from BE to adenocarcinoma. Over the past two decades, there have been many studies attempting to identify patients with BE and predict patients with a high risk of progression to adenocarcinoma[3-6].

In this review, the definition, mechanisms of produc-

Table 1 Phases of biomarker production

Phases of biomarker validation and development
Phase 1: Biomarkers of promise are identified based on application in other cancers and elucidation of novel pathways
Phase 2: Cross-sectional studies validate the biomarker of interest to be sufficiently discriminatory and biomarker assays are standardized
Phase 3: Case-control studies with a retrospective but longitudinal design confirm the biomarker is expressed before the development of cancer
Phase 4: Prospective longitudinal studies avoid biases associated with case-control studies
Phase 5: Population-based studies show the impact of biomarker detection on disease burden and cancer control

tion, and types of biomarker in patients with BE will be summarized.

DEFINITION OF BIOMARKERS

The biomarker

A biological marker affords an indication of the condition or disease, whether normal or abnormal. It is found in the blood, body fluids, and tissues. Moreover, a biomarker may be used for assessment of the response of the body to treatment of a disease or condition[7].

Phases of biomarker identification and validation

Biomarker discovery has to pass through 5 to 6 phases before clinical application (Table 1). Phases 4, 5 and 6 present a significant challenge because of the required large sample sizes, long follow-up and high costs[8].

TYPES OF BIOMARKERS IN PATIENTS WITH BE

Genomic instability

The similarity of the genetic patterns of BE and EAC demonstrated by DNA microarray studies supported the hypothesis that BE is a step preceding EAC. The genomic instability has been shown to be a poor prognostic marker in BE patients. Chromosomal alterations, deletions, point mutations, methylation abnormalities, and loss of heterozygosity (LOH) are the main indications of genomic instability in patients with BE[9-11].

DNA abnormalities

DNA abnormalities, *e.g.*, aneuploidy or tetraploidy, assessed by flow cytometry, can be used as predictive markers in patients with BE with no or low grade dysplasia[12,13]. LOH represents the loss of normal function of one allele of a gene in which the other allele was already inactivated. In a long-term follow-up study of BE patients, a panel combining 9p LOH, 17p LOH in addition to aneuploidy and tetraploidy was a strong predictor of EAC[14].

Abnormalities of tumor loci

An important predictor of risk of dysplasia and EAC in patients with BE is LOH for p53. LOH for p53 was shown to be associated with a 16-fold increase in the risk of progression to cancer[15]. However, in another study, in patients with non-dysplastic BE, only 32.4% of patients with progression showed overexpression of p53 in their initial biopsy[16]. Furthermore, alteration of APC, a regulator of the WNT pathway, by methylation[17] and LOH[18] were found in patients with BE with a positive predictive value.

Epigenetics

Epigenetics entails post-transcriptional silencing of specific genes without a change in the DNA sequence. A variety of mechanisms are involved, including methylation and acetylation. It has been shown that hypermethylation and loss of p16, are independently associated with an increased risk of progression from intestinal metaplasia (IM) to high-grade dysplasia (HGD)[19,20].

The p16 methylation was shown to be highly prevalent in patients with BE (34%-66%)[17,19,21]. Moreover, in a multicenter study, a panel of 8 genes (*p16*, *RUNX3*, *HPP1*, *NELL1*, *TAC1*, *SST*, *AKAP12*, and *CDH13*), was used to predict the risk of progression in patients with BE. In this study, 195 patients were included and sensitivities for prediction of progression approached 50%[22].

Cell cycle predictors

A dysregulated cell cycle may lead to accumulation of genetic aberrations in most cancer cells. Cyclins are cell cycle regulator proteins, and potentially useful biomarkers for progression. In patients with BE, cyclin D1 overexpression was shown to be associated with progression to EAC[23-26]. Further research in large groups of patients is needed to confirm the predictive values of cyclins.

Proliferation abnormalities

The association between increasing proliferation and worsening of dysplasia in BE was shown in many studies[26-28], while other studies found no association[29,30]. Researchers explained the discrepancies between there results by the use different techniques, the different histological pattern between columnar and squamous epithelium, and the use of different proliferative indices. One of the important markers of cellular proliferation is Ki67. However, in a long follow-up study, Ki67-positive proliferative fractions were not associated with risk of progression[31]. Further larger studies with standardized techniques are needed to measure proliferation.

Clonal diversity in BE

Genetic instabilities may lead to multiple distinct clones. The coexistence of multiple distinct clones is called clonal diversity. In patients with BE, clonal diversity measures

Table 2 Types of biomarkers in Barrett's esophagus

	Biomarker	Method	Remarks	Ref.
Diagnostic	TFF3	IHC	To screen asymptomatic patients for BE	[49,50]
	Chromosome 7 and 17 changes	IDKA and FISH	Early stages of BE	[52]
	8q24 (*C-MYC*), 17q12 (*HER2*), and 20q13 changes	FISH	Early stages of BE	[53]
	17q11.2 (*ERBB2*)	Microarray analysis	EAC	[54]
	Serum proteomic analysis	Mass spectrometry	EAC	[55]
Predictive	P16 allelic loss	FISH	Response to therapy	[56]
	DNA ploidy abnormalities	ICDA	Covariate value for recurrence	[57]
	HSP27	IHC	No response to therapy	[58]
	Ephrin B receptor	Microarray	Response to therapy in EAC	[59]
	Genetic polymorphism	qRT-PCR	Associated with clinical outcome	[60]
	P21	IHC	Correlated with better CTX response	[61]
	P53	IHC	Correlated with better CTX response	[62]
Progression markers	ERCC1	IHC	Predicts CTX resistance	[16]
	P53	IHC	Limited efficacy as a progression marker	[13,63]
	DNA abnormalities	Flow cytometry	High risk for progression to EAC	[13]
	LOH of 157p and 9p	Flow cytometry	Predict progression to EAC	[14]
	EGFR	IHC	Overexpression in HGD and EAC	[64]
	Cyclin A	IHC	Predicts progression to dysplasia	[65]
	Cyclin D1	IHC	Risk of Progression to EAC	[19]
	Hypermethylation of p16, RUNX2,HPP1	RT-PCR	Risk of progression to EAC/HGD	[22]
	8 gene methylation panel	RT-PCR	Predicts progression to EAC/HGD	[66]
Prognostic biomarkers	Cathepsin D,AKR1D10,AKR1C2 mRNA levels	Western blot, qRt-PCR	Dysregulation predicts progression to EAC/HGD	[67]
	DCK, PAPSS2, SIRT,TRIM44	RT-PCR, IHC	4 gene signature in EAC , predict 5 year survival	[56]
	P16 loss, C-MYC gain	FISH	Associated with therapy response	[68]
	ASS expression	Microarray	Low expression associated with metastases	[69]
	MicroRNA expression profile	Microarray, RT-PCR	Low level associated with worse prognosis in EAC	[70]
	Cyclin D1	IHC, FISH	Decreased survival	[71]
	EGFR	IHC	Decreased expression associated with decreased survival	[72]
	TGF-α	IHC, ISH	High level indicates progression and metastases	[73]
	TGF-β1	RT-PCR, ELISA	High expression associated with decreased survival	[73]
	APC	PCR	High level associated with decreased survival	[74]
	COX-2	IHC	Associated with metastases and recurrence	[75]
	Telomerase	Southern-blot and PCR	Associated with decreased survival	[76]
	VEGF	IHC	Associated with metastases and decreased survival	[77]
	Cadherin	IHC	Decreased level associated with decreased survival	[78]
	TIMP	IHC, PCR	Decreased level associated with decreased survival	[79]

ACIS: Automated cellular imaging system; ASS: Argininosuccinate synthase; APC: Adenomatous polyposis coli; BE: Barrett's esophagus; COX: Cyclooxygenase; DCK: Deoxycytidine kinase; DICM: Digital image cytometry; EAC: Esophageal adenocarcinoma; EGFR: Epidermal growth factor receptor; ELISA: Enzymelinked immunosorbent assay; FISH: Fluorescence in-situ-hybridization; ICDA: Image cytometric DNA analysis; HSP27: Heat-shock protein 27; IHC: Immunohistochemistry; LOH: Loss of heterozygosity; PAPSS2: 3'-phosphoadenosine 5'-phosphosulfate synthase 2; PCR: Polymerase chain reaction; qRT: Quantitative reverse transcriptase; MLPA: Multiplex ligation dependent probe amplification; NF-κB: Nuclear factor kappa B; SIRT2: Sirtuin 2; SNP: Single nucleotide polymorphism; TFF3: Trefoil factor 3; TGF: Transforming growth factor; TIMP: Tissue inhibitors of metalloproteinases; TRIM44: Tripartite motifcontaining 44; uPA: Urokinase-type plasminogen activator; VEGF: Vascular endothelial growth factor.

were strong predictors of progression[32]. However, the complicated methodology limited the use of clonal diversity as a predictive marker.

Mitochondrial DNA

Mitochondrial DNA (mtDNA) has been implicated in the process of carcinogenesis[33]. mtDNA mutations were found in 53% of patients with BE without dysplasia[32]. In patients with BE, deletion of the mitochondrial genome (4977 bp) was found in 15.4% in IM, 40% in low-grade dysplasia, 69.2% in HGD, and 90% in paratumoral tissue[34].

FLUORESCENCE *IN-SITU* HYBRIDIZATION

Fluorescence *in situ* hybridization (FISH) is a technique which detects DNA content and loci abnormalities in the cells by fluorescent-tagged DNA probes. FISH can detect aneusomy (abnormalities of chromosome copy number), deletion, duplication, amplification and translocation at tumor suppressor loci and protooncogene loci.

In patients with BE, FISH was used to detect genetic abnormalities by investigators in different studies from multiple centers[35-39]. Detection of dysplasia in BE and identification of HGD and EAC using the FISH 4-probe set has been shown to have a reasonable sensitivity (84%-93%) and specificity (93%)[39]. In another multicenter study, polysomy detected by FISH has been shown to predict risk of progression to HGD/EAC[40].

CLASSIFICATION OF BIOMARKERS OF BE

Biomarkers of BE can be classified into 4 groups: (1)

diagnostic biomarkers; (2) biomarkers of progression; (3) predictive biomarkers; and (4) prognostic biomarkers. This classification is based on the previous intensive research, and review articles[6,41-43] (Table 2).

Diagnostic biomarkers

Diagnostic biomarkers indicate the presence of disease. The histochemical analysis of biopsies of the gastro-esophageal junction remains the conventional approach for detection and diagnosis of BE. In patients with asymptomatic BE, trefoil factor 3 combined with a non-invasive diagnostic technique has been investigated with promising results in the screening of these patients[44,45]. Further validation and assessment are needed to confirm the results of these studies.

Progression biomarkers

The degree of dysplasia in obtained biopsies is the main marker of progression of BE, although there is much intra- and inter-observer errors[46-48]. The most promising biomarkers are minichromosome maintenance 2 (MCM2) expression pattern and LOH on distinct gene loci, especially at 17p. The cost and intensive laboratory time limit the use of these markers in clinical practice.

Predictive biomarkers

These biomarkers predict the response to therapy. A limited number of predictive biomarkers are available (Table 2) and this category is in need of further intensified research.

Prognostic biomarkers

These biomarkers indicate overall survival and prognosis of EAC. The majority of biomarkers are in this category. Prognostic biomarkers include growth signals, insensitivity to growth inhibitory signals, markers of evasion of programmed cell death, limitless replicative potential (telomerase), markers of sustained angiogenesis, markers of invasion and metastasis, marker of tumor differentiation, and cancer-related inflammation (Table 2).

Biomarkers in the clinical field: problems and obstacles

Much work is needed to set up clinical trials of biomarkers as this requires cooperation between clinical researchers and experts in molecular techniques. Moreover, the validation of a biomarker passes through 5 phases and requires multicenter studies, with prohibitive costs and long-term follow-up.

The method of specimen collection is another challenge. While microarray studies require special equipment and may not be easy to access by clinical scientists, molecular profiling using formalin-fixed paraffin-embedded specimens is interesting to researchers because of easy availability of specimens. In patients with hepatocellular carcinoma, the use of large scale (> 6000) gene profiling resulted in high quality data even from specimens archived for as long as 24 years[49].

The lack of prospective controlled trials is another important problem attributed to high costs and the need for large sample sizes. Moreover, the lack of reproducibility of assays between laboratories represent another obstacle for identification of clinically useful cancer biomarkers[50]. The reanalysis of DNA microarray studies showed that the selection of patients had an impact on the predictor role of genes in prognosis[51]. Careful interpretation of biomarker studies is needed by using large datasets such as DNA microarray repositories.

CONCLUSION

A biomarker for BE should help in population screening, improve the surveillance of patients with BE, and identify the prognostic groups and best therapy once EAC develops. Many biomarkers have been intensively studied and accurately predict the progress of BE to EAC. The MCM2 expression pattern, LOH on distinct gene loci, especially at 17p, hypermethylation of p16 and the expression pattern of P53 are promising markers especially for progression of the disease. Important prognostic biomarkers include cyclin D1, Ki-67, transforming growth factor-α, adenomatous polyposis coli, cyclooxygenase-2, telomerase and vascular endothelial growth factor. Till now, no biomarker has been able to replace the current gold standard of dysplasia in routine clinical practice. Panels of biomarkers seem to be better in predicting progression more accurately. The issue of costs and practicality of biomarkers should be considered before research is performed. A model incorporating clinical data and biomarkers will be promising and can accurately predict the risk of progression, prognosis or response to therapy. Similar models have been used in other cancers and diseases such as the Nottingham prognostic index for breast cancer and MELD score for liver disease. After generation and validation of such a model, it should then be rigorously validated in a large cohort of patients in a prospective fashion.

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TOPIC HIGHLIGHT

WJGP 5th Anniversary Special Issues (6): Crohn's disease

Role of bowel ultrasound in the management of postoperative Crohn's disease

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Abstract

The use of biological and immunosuppressive therapy in Crohn's disease (CD) changed favorably the course of the disease and is currently suggested in the prevention of clinical recurrence. Symptomatic exacerbation is a feature of the natural course of the disease. Endoscopic recurrence may occur earlier than clinical manifestations and its rate is still high ever since the first year after surgery. The severity of mucosal lesions is highly predictive of a new flare of the disease so that the early detection of recurrence warrants strong therapeutic changes or a closer monitoring of the case. Endoscopy is at present the gold-standard technique for the diagnosis and grading of recurrence severity, but is poorly accepted by patients for its invasiveness. A simple and easy repeatable examination able to detect early signs of recurrence could be useful in the follow-up as an alternative or as a backing in the choice of the right timing for endoscopy in questionable cases. The use of bowel ultrasound (B-US) in the management of CD has grown in the past twenty years. Its accuracy in the real time detection of the disease and its complications, known since the 80's, together with the non-invasiveness, low cost and wide availability of the technique have influenced the extension of its clinical use in many referral centers in Europe. The latest generation of ultrasound scanners allows a precise and reproducible morphological assessment of the intestinal tract and the surrounding tissues and enables a complete evaluation of the disease. This review analyzes the literature history about B-US in the diagnosis of postoperative recurrence of CD and outlines the clinical implications of its use. Published works confirm a very good accuracy of B-US in the diagnosis of CD recurrence compared to endoscopy, also in the early phase. B-US shows a good correlation with Rutgeert's score grading, but does not prove significant association with C-reactive protein or CD Activity Index values. A wider use of B-US in the daily practice could allow to set a prompt diagnosis and an earlier and targeted treatment, probably sparing more invasive tests.

Key words: Ultrasound; Endoscopy; Postoperative; Crohn's disease; Recurrence

Core tip: In the recent years, after the introduction of new drugs, prevention of recurrence is one of the emerging issues in the management of Crohn's disease because a more aggressive and earlier therapy is supposed to change the clinical course of the disease. Endoscopy, that is presently the standard reference for the diagnosis, is not well tolerated by patients. To asses pre-clinical signs of recurrence a non-invasive alternative is needed. Magnetic resonance imaging shows accurate results but with high costs and low availability. Bowel ultrasound can detect early specific signs of recurrence. Advantages, limits and clinical implications of the technique are discussed below.

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INTRODUCTION

The therapeutic management of Crohn's disease (CD) patients is an open challenge. The correct use of steroids, antibiotics, immunomodulators and biological therapies requires an appropriate timing in the decision making process. From this point of view an early diagnosis of postoperative recurrence is extremely important in order to identify patients with a more aggressive course and to address the correct therapeutic choice. Recurrence is endoscopically present in around 70% of patients at 1 year after surgery. Early endoscopic signs of recurrence have been detected even three months after surgery and the severity of mucosal lesions is highly predictive of future clinical manifestations of the disease[1,2].

Endoscopy is at present the gold-standard for the diagnosis of recurrence but less invasive, repeatable techniques would fit better to follow the evolution of chronic disease if they showed comparable results. The use of computed tomography (CT) should be limited because of its biological invasiveness while magnetic resonance (MR) can not be carried out routinely for its substantial costs and inadequate availability.

Starting from the first reports in the 80's on the possibility of detecting inflammatory bowel diseases using ultrasounds, the role of this technique in characterizing inflammatory bowel disease (IBD) in terms of extension, activity and complications compared to radiology or endoscopy has steadily increased[3-8].

In the last decade the continuous improvement in ultrasound technology enabled a better definition of the bowel wall morphology. The addition of color-power doppler, oral or intravenous contrast to advanced ultrasound (US) technical equipment made it possible to distinguish fibrotic from inflammatory involvement of the intestinal tract, phlegmons from abscesses and to select a portion of patients at increased surgical risk or with optimal response to new pharmacological approaches[9-13].

Advantages and limits of the technique and the technical aspects of potential impact on clinical practice are discussed below.

LITERATURE ANALYSIS

All the studies available in literature define post-surgical US recurrence as an increased bowel wall thickness at the anastomosis level and the majority of them correlates US findings with endoscopy. Major obstacles to a correct interpretation of the literature are due to a significant heterogeneity in the studies' design (different reference standards and variability in the timing of procedures), in technical aspects (different cut-offs for bowel wall thickness, BWT) and in the use of additional technical equipment (Power Doppler, Enteral or Intravenous Contrast Agents).

Since 1986 DiCandio *et al*[14] described the possibility of detecting post-surgical recurrence using transabdominal US compared to contrast radiography and endoscopy. His pioneering work on 32 patients showed a good sensitivity (82%) and an excellent specificity (100%) of the technique with an overall accuracy of 93.7%. In this study the possibility to distinguish between inflammatory and neoplastic lesions is shown through a structural study of the bowel wall, paying particular attention to the integrity of its layers[14].

In 1998 Andreoli studied the US detection rate of CD recurrence in 47 patients who underwent terminal ileum resection for CD using endoscopy at the anastomotic site as the gold standard. Bowel US sensitivity was 81%, specificity 86% and the overall accuracy 83%. The authors suggest to perform US in case of clinical suspected recurrence, reserving ileocolonscopy to negative or uncertain cases[15].

In 2001 and 2004 two studies have been published on the role of ultrasonography in the detection of recurrence after conservative surgery (strictureplastic and/or miniresections)[16,17]. Thickness and echopattern (the sequence of layers that constitute the sonographic appearance of the intestinal wall) of the diseased wall were considered before and 6 mo after surgery in patients with ileal stictures in order to understand if these characteristics and their postoperative behavior have a prognostic value. Both thickness and echopattern, in different measure, are relevant in order to reliably predict recurrence (hazards ratio 8.8 and 4.1 respectively).

A possible role of US as a predictor of endoscopic recurrence has been evaluated by Orlando *et al*[18] in 2006. Looking for the best calprotectin cut-off to assess recurrence, 50 resected patients were studied with US and fecal calprotectin every three months after surgery. Endoscopy was performed at one year. US sensitivity with a 5 mm BWT cut-off was 26% and specificity 90%. The best calprotectin cut-off value to predict the highest number of endoscopic recurrences was > 200 mg/L (sensitivity 63% and specificity 75%). Considering such a high specificity of US, the authors suggest that a positive ultrasound 3 mo after surgery, may be an indication to colonoscopy. In case of US negative, faecal calprotectin with a cut-off value of 200 mg/L could be a useful tool in order to decide if performing colonoscopy in asymptomatic patients.

In the study of Biancone *et al*[19] Bowel US was performed with oral contrast (small intestine contrast ultrasound - SICUS). Twenty-two asymptomatic patients, prospectively followed after surgery, underwent clinical controls every 3 mo and SICUS, wireless capsule endoscopy (WCE) and colonoscopy 1 year after surgery. Seventeen patients underwent all the 3 procedures. SICUS showed 100% sensitivity, 0% specificity (16 TPs, 1 FP), whereas WCE 100% sensitivity, 100% specificity (16 TPs, 1 TN). The small serie was then split in smaller subgroups. Considering only neo-terminal ileum recurrence and excluding patients in which disease was limited to the anastomosis the sensibility was 86% and specificity 33 %. In a very small subgroup (10 patients) SICUS and WCE were performed at 3, 6 and 12 mo. SICUS identified four of the nine WCE positive at month 3. At month 6, eight of the nine WCE positive were detected by SICUS. No

significant correlation between BWT and Rutgeert's score was found.

A part of a long term prospective follow up study on severity of CD recurrence after ileal resection published in 2010 by Pallotta *et al*[20] reports on 58 CD patients scheduled to SICUS and ileocolonoscopy at 6 mo regular intervals after surgery. Ileocolonoscopy was performed within 2 wk from SICUS. Bowel wall thickness at the anastomosis site was measured and it correlated with the anastomotic recurrence degree sec. Rutgeerts. SICUS could detect extension of intramural lesions even in patients with tight anastomotic stenosis.

In 2010, Onali *et al*[21] performed a longitudinal prospective study in 25 patients 3 years after surgery using oral contrast US and obtaining a very good correlation between SICUS and endoscopy. The correspondence of SICUS detected lesions with Rutgeert's grade was moderate and the attempt of identifying a bowel wall thickness value predictive for clinical recurrence did not reach statistical significance[21].

Between 2006 and 2010 other four prospective studies comparing US performance with endoscopy have been published[22-25]. In these studies sensitivity varies from 79% to 92% and specificity from 20% to 95%. In two of them oral contrast was used[23,24]. For all of them ileocolonoscopy was the reference standard and bowel wall thickness (> 3 mm) the only pathological feature considered. In one paper Doppler findings were considered, slightly strengthening the accuracy only in moderate-severe recurrence and with no impact on recurrence detection[25]. Bowel wall thickness was compared with Rutgeerts' score obtaining a good correlation between ultrasonographic findings and endoscopic lesions. Using a cut-off of 5 mm for bowel wall thickness mild from severe disease can be distinguished. No significant correlations between CD activity index (CDAI) and SICUS where found[24], while SICUS showed a higher sensitivity and specificity in detecting recurrence compared to CRP and CDAI values[23].

The use of intravenous contrast enhancement ultrasonography (CEUS) to emphasize B-US findings was reported by Paredes *et al*[26] in a study on postoperative recurrence of CD. The sample size of the study is consistent (60 patients) and the interval between ileocolonoscopy and CEUS was 3 d only. The study considered bowel wall thickness (cut-off 3-5 mm recurrence present, > 5 mm moderate-severe), color doppler vascularity (subjectively graded) and CEUS. The authors quantify ultrasonographic activity, with a software processing of the difference in brightness of contrast enhancement maximum uptake and the baseline and worked out a US activity score that correlates with Rutgeert's degree of severity. B-US sensitivity rises with CEUS from 89.8% to 98% while specificity keeps 81%.

In the same year Cammarota *et al*[27] published the largest retrospective study on the subject and investigate in particular the possible predictive role of BWT on surgical recurrence. All the patients included (196) were followed for 114 mo on average and the rate of surgical recurrence was 20.4%. Bowel US was performed 6-15 mo after surgery; bowel wall thickness > 3 mm was predictive of surgical recurrence. Moreover the authors describe an increased percentage of surgical recurrence in higher values of BWT at 1 year after surgery[27].

CONCLUSION

Several studies have been performed on bowel ultrasound and post-surgical recurrence in CD. Although most of them have a small sample size and different study designs, a very good correspondence between US and ileocolonoscopy is reported even in the early stages after surgery[18,24]. Bowel wall thickness is the main US parameter in the detection of recurrence. The majority of the studies compare ultrasonografic with endoscopic findings and BWT values > 3 mm shows, except in two cases[18,19], high percentage of sensibility and specificity (until 100% both) in identifying recurrence[14,15,20-26]. Some studies demonstrate also a correlation between BWT values (> 5 mm) and Rutgeert's score severe disease grade[19-24].

Few studies consider the echopattern performance before and after surgery in addition to bowel wall thickness[16,17]. Morphological alterations of the echopattern are a relevant parameter in the follow-up of CD, and a good correspondence of different echopatterns with histologic findings has been shown[28]. Moreover the predictive value of different echopatterns on the relative risk of surgical treatment and the normalization of the echopattern after biologic therapy have been reported[9,13].

Despite the positive data supporting its use, this technique is not widespread and its use is substantially limited to some European countries. The main criticism raised by some authors is the supposed low reproducibility of the method.

Ultrasonography is by definition a subjective technique and its employement in the study of ileum and colon may be particularly difficult considering the scarcity of repere points, the high anatomical variability expecially in post-surgical patients and the presence of gas in the bowel which implies the use of graduated pressure to display the deepest loops. On this issue (the reproducibility of B-US in the evaluation of Crohn' disease) a multicenter study has been performed which brought together gastroenterologists sonographers and radiologists from six referral centers for inflammatory bowel diseases, including our group. We found in different clinical settings of CD a good k value concerning BWT (K = 0.72-1) and the presence of complications (K = 0.81-1)[29].

The performance of the examination, blinded, sequentially conducted by different operators, was preceded by a long theoretical comparison that led to the choice of parameters to be measured and methods of detection.

The results of this experience, combined with the well known positive characteristics of ultrasound (optimal tolerance, low invasiveness, low costs, wide availability) and the comparable accuracy values of B-US, CT and

MRI in different controlled settings of CD attest B-US as an added value in the clinical management of IBD[30-32].

In our opinion features needed for a correct use of B-US are an adequate learning curve, a good clinical knowledge in inflammatory bowel diseases and the basics of ultrasound technique. The use of B-US should be included in pathways of clinical management at different levels in the management of inflammatory bowel diseases (screening IBS-IBD, therapy monitoring, follow up of complications, emergency, young children) because it raises an efficient clinical work up and reduces the use of more expensive and invasive tests with similar results in terms of clinical impact[31,32].

It is conceivable that new technologies can improve the correspondence between imaging and the bowel wall morphology in intestinal inflammation. A wider confrontation among experienced operators on this and other interesting US parameters in B-US would be desirable.

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WJGP 5th Anniversary Special Issues (6): Crohn's disease

Quality of care in Crohn's disease

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Abstract

Crohn's disease (CD) is a chronic and progressive inflammatory disease of the intestine. Overall, healthcare delivery for patients with CD is not optimal at the present time and therefore needs improvement. There are evidences which suggest that there is a variation in the care provided to patients with CD by the inflammatory bowel disease (IBD) experts and community care providers. The delivery of healthcare for patients with CD is often complex and requires coordination between gastroenterologists/IBD specialist, gastrointestinal surgeon, radiologists and IBD nurses. In order to improve the quality of health care for patients with CD, there is need that we focus on large-scale, system-wide changes including creation of IBD comprehensive care units, provision to provide continuous care, efforts to standardize care, and education of the community practitioners.

Key words: Inflammatory bowel disease; Quality assurance; Quality indicators; Outcome; Comprehensive care units; Quality improvement

Core tip: Crohn's disease (CD) is a progressive inflammatory disease of the intestine. Overall, healthcare delivery for patients with CD is not optimal at the present time and therefore needs improvement; Despite availability of national and international practice guidelines, there is a variation in the care provided to patients with CD; There is a need to develop well defined quality indicators which assures delivery of adequate care of the disease.

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INTRODUCTION

Crohn's disease (CD) is a chronic and progressive inflammatory disease of the intestine, which occurs because of interaction between, immunological factors, environmental factors and gut microbiome[1].

At the onset of the disease, the majority of patients with CD while have ulcerations in the intestine, the course of the disease gets complicated with patients developing strictures and fistula in the intestine[2]. In a study including 297 patients with CD over 25 years, Louis *et al*[3] reported a change in the behavior of the disease in 46% patients from non-stricturing, non-penetrating to either stricturing (27%) or penetrating (29%) disease in the first 10 years of follow-up. Because of the progressive nature of the disease, patients with CD are more likely to require not only repeated hospitalization but also surgical interventions[4,5].

While majority of patients with CD generally present in third and fourth decade of their lives, approximately one fifth of them become symptomatic during childhood and nearly 5% of them even before 10 years of their age[6]. Failure to thrive, retardation in the linear growth and defect in bone formation are the major issues in pediatric patients with active CD. Even puberty gets delayed in children patients with CD. Therefore, induction of re-

mission of the disease and maintenance of remission before the onset of the puberty is essential for children patients with CD. A good control of inflammatory activity is required to prevent or even minimize the consequences of a missed pubertal growth spurt and the maintenance of pre-pubertal levels of sex hormones. Since more than 90% of the bone mass is attained during childhood and adolescence, inflammatory diseases during this period can affect bone development and may ultimately lead to osteopenia and make them susceptible to fractures[7].

Till a few years back, control of symptoms has been considered to be an end point of treatment of CD; over the past years however, healing of mucosal ulcerations has emerged as a major therapeutic goal for patients with CD[8-10]. There are now evidences which suggest that healing of mucosal ulcerations with anti-inflammatory/immunomodulators or biologicals has a potential for changing the natural history of the disease and the available primitive evidences suggest that there is reduction in the rate of hospitalization and requirement for surgery in those patients who attains mucosal healing[11].

The treatment of CD depends upon the activity (active phase, remission phase), location, extent and behaviour (inflammatory, stricturing, fistulizing) of the disease[1]. The treatment needs to be tailored for each patient. The choice of treatment is also influenced by well-known negative prognostic predictors of CD such as young age of onset, presence of extensive disease, stricturing disease, and positive smoking history[12].

Chronic disease management has become a significant focus for providing a quality and continuous care of these diseases in order to decrease their morbidity and mortality[13]. Care of chronic diseases requires a continuous and optimal care including use of newly discovered with proven value diagnostic or therapeutic strategies. The question arises, are we providing a standard and quality care to patients having chronic diseases? In a landmark study from US, based on review of medical records and telephonic interviews, has shown that only 57% of patients attending the outpatients clinic regularly receive recommended standard of care for a variety of acute and chronic illnesses[14]. This study has raised an important concern and further highlights the importance of delivering an evidence based care and preventative measures to patients with chronic diseases in order to decrease complications, hospitalizations, and death.

QUALITY OF CARE IN INFLAMMATORY BOWEL DISEASE IS SUBOPTIMAL

Since there is no definite cure for most patients with CD, the main objectives of treatment therefore include induction of remission and maintenance of remission; minimization of complications of the disease such as strictures, fistulae, osteoporosis, short-and long-term toxicities of the drugs; improvement in quality of life; decrease in number of hospitalizations and surgeries; and maintenance of linear growth in pediatric patients. The practice of many chronic diseases is generally guided by evidence based literature and on the guidelines of both International and national societies[12,15-17]. While there is some degree of variability amongst the guidelines, the essential components remain more or less similar, since such recommendations are based on the available evidences derived from a body of published literature. Since CD is a disease with heterogeneous characteristics, treatment is generally tailored or individualized for a particular patient[12,15-18].

There is a variability in the treatment provided by an expert and a general practitioner especially for diseases, which are heterogeneous in their clinical behavior and where treatment options and guidelines are still emerging[19]. A variability in the care of a particular disease provided by various physicians is regarded as an index for poor quality of care. In inflammatory bowel disease (IBD), there is evidence of a high degree of variation of care for both patients with UC and CD[19]. In order to develop quality indicators for care, it is therefore, critical to understand the current status of care of such diseases. If current practice varies widely and is not well standardized, it calls for standardization of treatment protocols.

In a survey on the management of CD by IBD experts and community care providers, Esrailian *et al*[19] reported that there was good agreement in the decision making of diagnostic testing between community care providers and the IBD experts. In the management decisions, there was significant disagreement between community care providers and IBD experts[19]. While most community care providers in this study believed that 5-aminosalicylate products were appropriate across a variety of presentations of CD, IBD experts were significantly less likely to endorse 5-ASA use in patients with CD. In contrast to 5-ASA results, experts and community providers generally agreed with each other on the use of immunomodulators, infliximab and antibiotics in CD. Furthermore, the differences existed not only between community care providers and IBD experts; there was marked differences in the management decisions taken by various IBD experts, especially with the use of immunomodulators in newly diagnosed CD and perianal fistulizing CD[19].

Another study including patients with CD and UC also suggested that patients with IBD often do not receive optimal medical therapy. The main points include suboptimal dosing of 5-ASA and immunosuppressive therapy, prolonged use of corticosteroids, underuse of immunosuppressive drugs, non-compliance to use of calcium and vitamin D, and inadequate screening for colorectal cancer[20].

QUALITY IMPROVEMENT AND QUALITY ASSURANCE

Quality improvement (QI) and quality assurance (QA) are now becoming essential components of public services including delivery of healthcare services. While quality improvement is used to describe the process of

Table 1 Measures to provide quality care to patient with Crohn's disease

Delivery of high quality, safe and integrated clinical care for IBD patients based on multi-disciplinary team called IBD Comprehensive Care Unit
Delivery of care at the local center and if needed with rapid access to more specialized IBD care center
Patient education and support
Care for IBD patients that is patient-centered, responsive to individual needs
Regular audit of the care provided and outcomes

IBD: Inflammatory bowel disease.

implementing evidence-based interventions to bridge the disparities currently present in various healthcare systems; quality assurance is defined as planned, systematic activities that are implemented to ensure that a level of performance is attained[21]. In any chronic disease process the three main objective of care include improvement in population health, improvement in patient's experience of care, and at the mimimal cost; all three together are defined as Triple Aim of the disease[22].

The essential building blocks for quality improvement efforts are the proper identification and implementation of effective quality indicators. These quality indicators are measurable elements of practice performance for which there is evidence or consensus that they may be applied to assess and improve the quality provided[23-25]. The types of quality indicators have been broadly categorized as structural measures, process measures and outcome measures. Structural measures are indicators to do with the structure of the health system such as staffing, equipment, and electronic medical records. Process indicators are the processes of providing care such as investigations, treatment, and interactions with patients. Outcomes indicators assess the outcome of patients such as quality of life, patient satisfaction, prophylactic vaccines, mortality and morbidity. While improvement in all categories of indicators is desirable, process measures have garnered the majority of the attention, as they are most easily modifiable.

EFFORTS TO IMPROVE QUALITY OF CARE

Health care measures such as use of electronic medical record systems, automated entry of diagnostic and therapeutic orders, decision support tool at the point of care, and routine measurement of and reporting on quality have been shown to improve the quality of care[14]. In 2004, with funding from the American Board of Pediatrics, a group of care providers started a "research and improvement network", focused on improving care for children and teens with CD[26]. ImproveCareNow (ICN) network invited care providers to form collaboration to record information from all the patient visits and the care they were providing to children with IBD[27]. With insitution of protocal based recording of care, the group observed an increase in the proportion of visits with complete disease classification, measurement of thiopurine methyltransferase (TPMT) before initiation of thiopurines, and patients receiving an initial thiopurine dose appropriate to their TPMT status. Furthermore, an increase in the proportion of patients either CD or UC having inactive disease on follow up was observed, suggesting a better care. The number of patients taking prednisolone also decreased[28]. With the similar changes in the practice at IBD center at Cincinnati Children's Hospital Medical Center, there was an increase in the clinical remission rate from 59% to 76% ($P < 0.05$), decrease in frequency of steroid use from 17% to 10% and an increase in patients having Short Pediatric Crohn's Disease Activity Index < 15 from 60% to 77%[29].

These preliminary studies from ICN are testimony that a large-scale pediatric IBD quality improvement network can change practice and improve the quality of care. The key measures required for the delivery of quality care to patients with CD is summarized in Table 1.

QUALITY INDICATORS FOR IBD

There is a lack of definitive guidelines on the measurement of quality indices in IBD. The American Gastroenterology Association has recommended 10 indices as a measurement of quality of care in IBD[30] (Table 2). Similarly, the Crohn's and Colitis Foundation of America have also proposed a questionnaire for the assessment of quality of care of patients with IBD[31,32] (Table 2).

In order to identify a set of quality indices, Calvet *et al*[33] conducted a two-round web-based survey including an expert panel of patient representatives ($n = 4$), nurses ($n = 7$), surgeons ($n = 2$) and physicians ($n = 18$) using Delphi consensus-based approach. The expert panel selected a core set of 56 QIs (including 12 structure, 20 process and 24 outcome related). Structure and process quality indicators highlighted the need for multidisciplinary management and continuity of care. The key outcome quality indices focused on the adequate prophylaxis of disease complication and drug adverse events, the need to monitor appropriateness of treatment and the need to reinforce patient autonomy by providing adequate information and facilitating the patients' participation in their own care. The panel also suggested that there should be an IBD team and the team should be consisted of gastroenterologists, radiologists, surgeons, endoscopists, IBD nurse, and stoma management specialists.

HOW TO IMPROVE QUALITY OF CARE: A CONCEPT OF IBD COMPREHENSIVE CARE UNIT

The care of CD requires a coordinated action of a number of health care professionals such as a gastroenterologists/IBD expert, gastrointestinal surgeon, radiologist, stoma care personel and well trained nurses. All of them can

Table 2 Quality of care indicators in inflammatory bowel disease

	Quality of care indicators
10 quality of care indicators by American Gastroenterology Association	IBD: type, anatomic location and activity all assessed IBD preventive care: corticosteroid sparing therapy IBD preventive care: corticosteroid related iatrogenic injury - bone loss assessment IBD preventive care: influenza immunization IBD preventive care: pneumococcal immunization Testing for latent tuberculosis before initiating anti-TNF therapy Assessment of hepatitis B virus before initiating anti-TNF therapy Testing for Clostridium difficile - inpatient measure Prophylaxis for venous thromboembolism - inpatient measure IBD preventive care: tobacco user - screening and cessation intervention
CCFA top 10 quality outcome indicators of IBD	Corticosteroid use Proportion of patients with steroid-free clinical remission for a 12-mo period Proportion of patients currently taking prednisone Number of days per month and year lost from school or work because of IBD Number of days hospitalized per year because of IBD Number of emergency room visits per year for IBD Proportion of patients with malnutrition Proportion of patients with anemia Proportion of patients with normal disease-targeted health-related quality of life Proportion of patients currently taking narcotic analgesics Proportion of patients with nighttime bowel movements or leakage Proportion of patients with incontinence in the past month

IBD: Inflammatory bowel disease; TNF: Tumor necrosis factor; TB: Tuberculosis.

form a IBD Comprehensive Care Unit (ICCU). While it is commonly accepted that ICCUs facilitate the provision of quality care to patients with IBD, a structure of ICCU is still not well defined[33].

The cost of implementing some of these quality measures is modest suggesting that substantial improvement is possible. Individuals at all levels from senior clinicians to administrative staff should be encouraged to identify areas of potential improvement in the quality of care. In all settings, quality indicators should be seen as a team effort of the practice as a whole. One of the important features of chronic disease care is to provide continuous care, such as from clinic to home, interval reminder and also in between appointment care.

ANTAGONISTIC VIEW

While most supports the view that providing a quality care is a essential element of healthcare delivery system, a few believes that the imposition of quality measures may disrupt the art of medicine and the precious minutes at an office visit may be lost in documentation rather than spending time in thoughtfully delivered health care[22,34].

PROVIDING QUALITY OF CARE IN RESOURCE LIMITED COUNTRIES

Providing quality care in resource limited countries is a real challenge. The barrier to impart quality of CD care in resource limited countries may mainly be structure related such lack of optimal number of IBD experts, lack of diagnostic facilities, and affordability and non-referral of patients to tertiary care centers.

CONCLUSION

The delivery of healthcare for patients with CD is often complex and requires coordination between gastroenterologists/IBD specialist, gastrointestinal surgeon, radiologists and IBD nurses. Overall, healthcare delivery for patients with CD may not be the optimal at the present time and therefore needs improvement. There are evidences which suggests that there is a variation in the care provided by the IBD expert and general practitioner. To make substantial improvements in the quality of health care available to all patients, there is need of making large-scale, system-wide changes.

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TOPIC HIGHLIGHT

WJGP 5th Anniversary Special Issues (9): Gastrointestinal bleeding

Diagnosis of gastrointestinal bleeding: A practical guide for clinicians

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Abstract

Gastrointestinal bleeding is a common problem encountered in the emergency department and in the primary care setting. Acute or overt gastrointestinal bleeding is visible in the form of hematemesis, melena or hematochezia. Chronic or occult gastrointestinal bleeding is not apparent to the patient and usually presents as positive fecal occult blood or iron deficiency anemia. Obscure gastrointestinal bleeding is recurrent bleeding when the source remains unidentified after upper endoscopy and colonoscopic evaluation and is usually from the small intestine. Accurate clinical diagnosis is crucial and guides definitive investigations and interventions. This review summarizes the overall diagnostic approach to gastrointestinal bleeding and provides a practical guide for clinicians.

Key words: Gastrointestinal hemorrhage; Diagnostic techniques; Endoscopy; Colonoscopy; Capsule endoscopy; Enteroscopy; Computed tomography; Angiography

Core tip: This review provides a practical diagnostic guide for clinicians who encounter patients with suspected gastrointestinal bleeding in the hospital and primary health care settings. Clinical presentations of gastrointestinal bleeding are classified as overt (acute), occult (chronic) or obscure and the corresponding diagnostic algorithms are illustrated through review of the key evidence and consensus guidelines. Upper endoscopy and colonoscopy are the mainstay of initial investigations. Angiography and radionuclide imaging are best suited for acute overt gastrointestinal (GI) bleeding. Capsule endoscopy and deep enteroscopy play significant roles in the diagnosis of obscure GI bleeding, usually from the small bowel.

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INTRODUCTION

Gastrointestinal (GI) bleeding is a common problem medical practitioners encounter in the emergency department and in the primary care setting[1]. Annual hospital admissions for GI bleeding in the United States and United Kingdom have been estimated at up to 150 patients per 100000 population with a mortality rate of 5%-10%[2-5]. While GI bleeding can be potentially life-threatening, it has been shown that many cases can be safely managed on an outpatient basis[6]. The accurate diagnosis of GI bleeding relies on prompt resuscitation, initial risk evaluation, provisional clinical diagnosis followed by appropriate definitive investigation which enables specific interventions. This review provides a practical diagnostic guide for clinicians who may encounter patients with suspected GI bleeding.

DEFINITIONS

Overt (acute) vs occult (chronic) vs obscure

Although GI bleeding can be a result of benign pathology, life-threatening hemorrhage, varices, ulceration and malignant neoplasms need to be considered and carefully excluded[7,8]. Given the wide range of underlying pathology and the differences in their appropriate diagnostic approach, it is crucial for clinicians to define the type of GI bleeding based on clinical presentation.

Depending on the rate of blood loss, GI bleeding can manifest in several forms and can be classified as overt, occult or obscure. Overt GI bleeding, otherwise known as acute GI bleeding, is visible and can present in the form of hematemesis, "coffee-ground" emesis, melena, or hematochezia. Occult or chronic GI bleeding as a result of microscopic hemorrhage can present as Hemoccult-positive stools with or without iron deficiency anemia[9,10]. The American Gastroenterological Association defines occult GI bleeding as the initial presentation of a positive fecal occult blood test (FOBT) result and/or iron-deficiency anemia when there is no evidence of visible blood loss to the patient or clinician[11]. Obscure GI bleeding refers to recurrent bleeding in which a source is not identified after upper endoscopy and colonoscopy. Obscure bleeding may be either overt or occult[10-12].

Upper vs lower

Upper GI bleeding includes hemorrhage originating from the esophagus to the ligament of Treitz, at the duodenojejunal flexure[13]. Lower GI bleeding is defined as bleeding that originates from a site distal to the ligament of Treitz[14]. In recent years upper GI bleeding has been redefined as bleeding above the ampulla of Vater within reach of an upper endoscopy; lower GI bleeding has been further subdivided into mid GI bleeding coming from the small bowel between the ampulla of Vater to the terminal ileum, and lower GI bleeding coming from the colon[11].

OVERT (ACUTE) GI BLEEDING

Epidemiology

Acute GI bleeding is a major cause of hospital admissions in the United States, which is estimated at 300000 patients annually[15]. Upper GI bleeding has an annual incidence that ranges from 40-150 episodes per 100000 persons and a morality rate of 6%-10%[16-18]; compared with lower GI bleeding which has an annual incidence ranging from 20-27 episodes per 100000 persons and a mortality rate of 4%-10%[19,20]. Acute GI bleeding is more common in men than women and its prevalence increases with age[13,21].

Etiology and pathophysiology

Acute upper GI bleeding may originate in the esophagus, stomach, and duodenum. Upper GI bleeding can be categorized based upon anatomic and pathophysiologic factors: ulcerative, vascular, traumatic, iatrogenic, tumors, portal hypertension. The commonest causes of acute upper GI bleeding are peptic ulcer disease including from the use of aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs), variceal hemorrhage, Mallory-Weiss tear and neoplasms including gastric cancers[8]. Other relatively common causes include esophagitis, erosive gastritis/duodenitis, vascular ectasias and Dieulafoy's lesions[22]. Significant geographical variations in pathophysiology exist for esophageal varices and peptic ulceration between the East and the West, with East Asians having a stronger association with non-alcoholic cirrhosis and helicobacter pylori as their respective etiologies which generally have a more favorable prognosis[23,24]. However, esophageal varices and peptic ulcer disease are nevertheless major causes of upper GI bleeding in both Eastern and Western societies[24,25].

Acute lower GI bleeding may originate in the small bowel, colon or rectum[21]. The causes of acute lower GI bleeding may also be grouped into categories based on the pathophysiology: vascular, inflammatory, neoplastic, traumatic and iatrogenic. Common causes of lower GI bleeding are diverticular disease, angiodysplasia or angiectasia, neoplasms including colorectal cancer, colitis including Crohn's disease and ulcerative colitis, and benign anorectal lesions such as hemorrhoids, anal fissures and rectal ulcers[8].

In the special setting where the patient is known to have an abdominal aortic aneurysm or an aortic graft, acute GI bleeding should be considered secondary to aortoenteric fistula until proven otherwise[26].

Initial evaluation

Rapid assessment and resuscitation should precede diagnostic evaluation in unstable patients with acute severe bleeding[27]. Once hemodynamic stability is assured, patients should be evaluated for the immediate risk of rebleeding and complications, as well as the underlying source of bleeding. For acute upper GI bleeding, risk scores such as the Rockall Score and Glasgow Blatch-

ford Score (GBS) have been developed and validated[6,28]. Patients with minimal or intermittent bleeding who are stratified as low risk can be evaluated in an outpatient setting, allowing more effective utilization of limited hospital in-patient resources[19]. While the Rockall score uses endoscopic findings, the GBS is based upon the patient' s clinical presentation such as systolic blood pressure, pulse, presence of melena, syncope, hepatic disease, cardiac failure and laboratory parameters such as blood urea nitrogen and hemoglobin. A meta-analysis found that a GBS of zero decreases the likelihood of requiring urgent intervention (likelihood ratio 0.02, 95%CI: 0-0.05)[4]. Therefore, the GBS may be best suited for initial risk evaluation of suspected acute upper GI bleeding, such as in the emergency department setting.

As in the diagnosis of any disease, the clinical history, physical examination and initial laboratory findings are crucial in determining the likely sources of bleeding which would help direct the appropriate definitive investigation and intervention. A medication history here is particularly important, especially on the use of aspirin and other NSAIDs.

Clinical presentation

Upper GI bleeding usually presents with hematemesis (vomiting of fresh blood), "coffee-ground" emesis (vomiting of dark altered blood), and/or melena (black tarry stools). Hematochezia (passing of red blood from rectum) usually indicates bleeding from the lower GI tract, but can occasionally be the presentation for a briskly bleeding upper GI source[9]. The presence of frank bloody emesis suggests more active and severe bleeding in comparison to coffee-ground emesis[29]. Variceal hemorrhage is life threatening and should be a major consideration in diagnosis as it accounts for up to 30% of all cases of acute upper GI bleeding and up to 90% in patients with liver cirrhosis[30].

Lower GI bleeding classically presents with hematochezia, however bleeding from the right colon or the small intestine can present with melena[31]. Bleeding from the left side of the colon tends to present bright red in color, whereas bleeding from the right side of the colon often appears dark or maroon-colored and may be mixed with stool[31].

Other presentations which can accompany both upper and lower GI bleeding include hemodynamic instability, abdominal pain and symptoms of anemia such as lethargy, fatigue, syncope and angina[21]. Patients with acute bleeding usually have normocytic red blood cells. Microcytic red blood cells or iron deficiency anemia suggests chronic bleeding. In contrast to patients with acute upper GI bleeding, patients with acute lower GI bleeding and normal renal perfusion usually have a normal blood urea nitrogen-to-creatinine or urea-to-creatinine ratio[32]. In general, anatomic and vascular causes of bleeding present with painless, large-volume blood loss, whereas inflammatory causes of bleeding are associated with diarrhoea and abdominal pain[33].

When patients with known abdominal aortic aneurysm or aortic graft present with above symptoms of GI bleeding, aortoenteric fistula most commonly at the duodenum should be strongly suspected. In this case, urgent computed tomography (CT) abdomen or CT angiogram is indicated to look for loss of tissue plane between the aorta and duodenum, contrast extravasation and the presence of gas indicating graft infection. Upper endoscopy prior to surgical intervention may help exclude other diagnoses when CT findings are not definitive[26,34]. The details of these investigations are discussed later in this review.

Investigations

Options for the investigation of acute GI bleeding include upper endoscopy and/or colonoscopy, nuclear scintigraphy, CT angiogram and catheter angiography. The investigation of choice would be guided by the suspected location of bleeding (upper *vs* lower GI) based on clinical presentation. In most circumstances, the standard of care for the initial diagnostic evaluation of suspected acute GI bleeding is urgent upper endoscopy and/or colonoscopy, as recommended by guidelines from the American College of Gastroenterology and the 2010 International Consensus Recommendations[20,27]. As investigations are being planned, infusions of proton pump inhibitor or octreotide should be initiated for suspected bleeding peptic ulcer and varices respectively[27,30].

Upper endoscopy

In patients with acute upper GI bleeding, upper endoscopy is considered the investigation of choice[35]. Early upper endoscopy within 24 h of presentation is recommended in most patients with acute upper GI bleeding to confirm diagnosis and has the benefit of targeted endoscopic treatment (Figure 1), resulting in reduced morbidity, hospital length of stay, risk of recurrent bleeding and the need for surgery[27]. Endoscopic evacuation of hematoma or blood clot may enable visualization of underlying pathology such as a visible vessel in a peptic ulcer and allows directed endoscopic hemostatic therapy[36,37]. The reported sensitivity and specificity of endoscopy for upper gastroduodenal bleeding are 92%-98% and 30%-100%, respectively[38]. Risks of upper endoscopy include aspiration, side-effects from sedation, perforation, and increased bleeding while attempting therapeutic intervention. The airway should be secured by endotracheal intubation in the case of massive upper GI bleeding.

The use of nasogastric-tube insertion and gastric lavage in all patients with suspected upper GI bleeding is controversial and studies have failed to demonstrate a benefit in clinical outcomes[39,40]. The use of prokinetics such as erythromycin and metoclopramide as a single dose before upper endoscopy promotes gastric emptying and clearance of blood, clots and food. Two meta-analyses have demonstrated the use of a prokinetic agent improved visibility at endoscopy and significantly reduced the need for repeat endoscopy[41,42]. In particular, the use

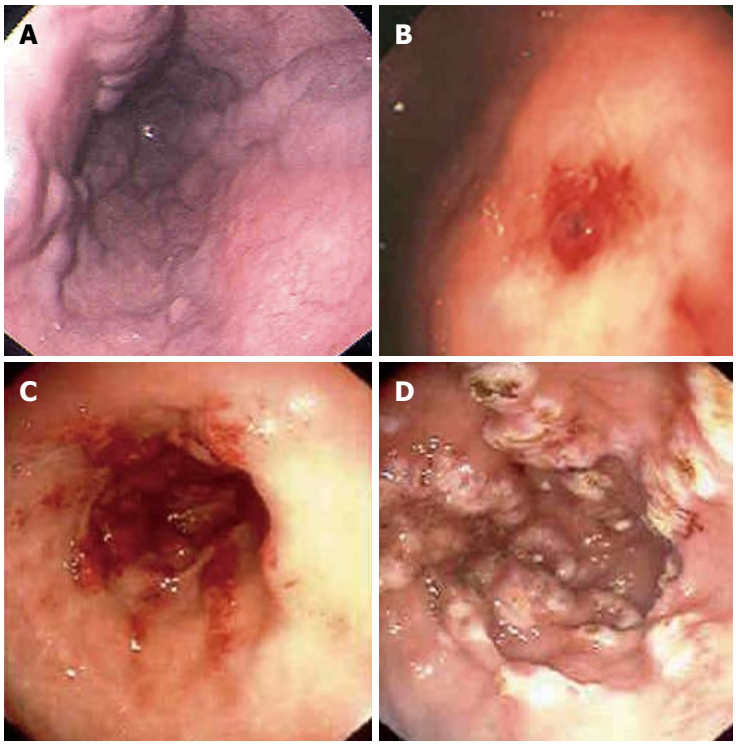

Figure 1 Upper endoscopic findings in patients with suspected upper gastrointestinal bleeding. Esophageal varices (A), Dieulafoy's lesion in the stomach (B), gastric antral vascular ectasia (watermelon stomach) in the antrum of the stomach pre and post argon plasma coagulation therapy (C, D).

of erythromycin was associated with a decrease in the amount of blood in the stomach, reduced amount of blood transfusion and shorter length of hospital stay[42]. Therefore prokinetics such as erythromycin before upper endoscopy should be recommended for patients with major bleeding who are expected to have large amount of blood in the stomach.

The practice of routine second look endoscopy after hemostasis is achieved on first endoscopy remains controversial. Two meta-analyses of randomized controlled trials have shown that second look endoscopy significantly reduced peptic ulcer rebleeding but did not improve overall mortality[43,44]. Due to the relatively small number of subjects studied, suboptimal hemostatic measures used and the lack of proton pump inhibitor use in those trials, the 2010 International Consensus Recommendations did not recommend routine use of second look endoscopy but stated it may be useful in selected patients with high risk of re-bleeding[27]. This should be considered particularly when there are concerns of suboptimal prior endoscopy and potential missed lesions.

In cases of acute upper GI bleeding where upper endoscopy is non-diagnostic in which a bleeding site cannot be identified or treated, the next investigation depends on the patient's hemodynamic stability. If the patient is unstable with large volume upper GI blood loss, patient should proceed to urgent surgery, such as an exploration and partial gastrectomy for uncontrolled bleeding gastric ulcer[9]. Intraoperative endoscopy may be a useful adjunct during surgery to help localize the source of bleeding[45,46]. If the patient is hemodynamically stable with low volume bleeding, repeat endoscopy may be considered. Colonoscopy should also be considered in the setting of melena to exclude a right-sided colonic source of bleeding, as discussed later.

Further imaging should be considered after non-diagnostic upper endoscopy with or without colonoscopy and the options include CT angiography, catheter angiography and nuclear scintigraphy[38], all of which are discussed separately in later sections of this review. Upper GI barium studies are contraindicated in the setting of acute upper GI bleeding because they may interfere with subsequent investigations or surgery[22], and due to the risk of barium peritonitis if there is a pre-existing perforation of the bowel wall[47].

Colonoscopy

In acute lower GI bleeding, the diagnostic approach is somewhat more variable. Colonoscopy and CT angiogram are the two diagnostic tools of choice for evaluation of acute lower GI bleeding[15]. The American College of Gastroenterology guidelines suggest that colonoscopy should be the first-line diagnostic modality for evaluation and treatment of lower GI bleeding[20]. Studies have indicated that colonoscopy identifies definitive bleeding sites (Figure 2) in 45%-90% of patients[48]. Advantages of colonoscopy include direct visualization, access to tissue biopsy and endoscopic hemostatic therapy, and as an initial diagnostic test has a higher sensitivity[15,49]. However, there are several limitations to colonoscopy in the setting of acute lower GI bleeding, including potential inadequate bowel preparation, the inability to evaluate most of the small bowel, as well as risks associated with sedation, perforation and bleeding similar to upper endoscopy[50]. In patients with inadequate bowel preparation, the sensitivity drops significantly and successful treatment may only be possible in as few as 21% of patients in the acute setting[51]. It has been advocated that urgent colonoscopy

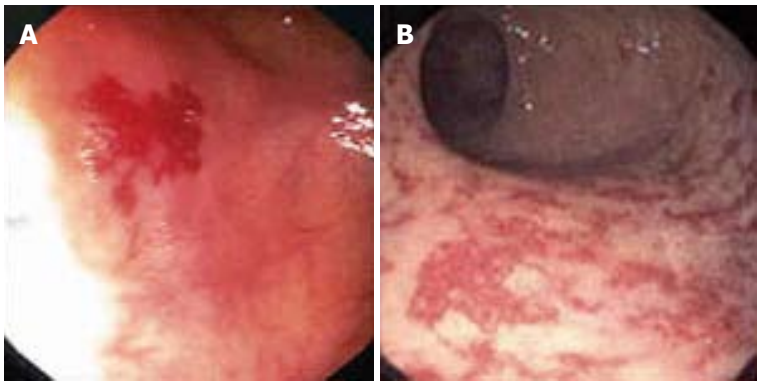

Figure 2 Colonoscopic findings in patients with suspected lower gastrointestinal bleeding. Colonic angiodysplasia (A) and radiation proctopathy (B).

in this setting should be preceded by a rapid purge with isotonic colonic lavage 4-6 liters orally until the effluent passed is diluted pink in color. This rapid purge may require the use of a nasogastric tube and a prokinetic agent such as metoclopramide. This is based on the findings that blood or stool in the colon can obscure the bleeding source during urgent colonoscopy[51,52].

It is recommended by the American College of Radiology that colonoscopy be utilized as the initial modality in hemodynamically stable patients (allowing for adequate bowel preparation) and angiography in those are who are hemodynamically unstable with massive lower GI bleeding[53]. It should be noted that colonoscopy is also indicated in the evaluation of patients presenting with melena who have negative upper endoscopy to exclude a right-sided colonic source of bleeding.

In cases where the source of bleeding is unidentified after upper endoscopy and/or colonoscopy, the utilization of subsequent diagnostic modalities should be guided by clinical presentation, hemodynamic stability and local expertise with the individual tests. No large randomized trials have demonstrated superiority of a particular strategy. The next section will outline the diagnostic use of CT angiography, catheter angiography and radionuclide imaging in acute GI bleeding.

CT angiography

CT angiography requires the rate of ongoing arterial bleeding to be at least 0.5 mL/min to reliably show extravasation of contrast into the bowel lumen to identify a bleeding site[54]. A systematic review of the diagnostic accuracy of CT angiography demonstrated a sensitivity of 86% and specificity of 95% in the evaluation of patients with acute GI bleeding[55]. The potential advantages of CT angiogram in diagnosis of acute GI bleeding include its minimally invasive nature and its wider availability in comparison to catheter angiography[38]. It can also demonstrate neoplasms or vascular malformations and provide evidence of recent bleeding, such as hyperdense blood in bowel lumen[38,56]. Active GI bleeding is diagnosed by extravasation of contrast into the bowel lumen, which appears as an area of high attenuation on the arterial phase scan which increases on the venous phase scan (Figure 3A-D). By demonstrating the precise site of bleeding and the underlying etiology, CT angiography is useful for directing and planning definitive treatment whether it be through endoscopy, catheter angiography or surgery[57]. If the gastrointestinal bleeding is intermittent and the initial CT is negative, a repeat CT angiogram can be performed when rebleeding occurs[58].

Disadvantages of CT angiography is the lack of therapeutic capability, risk of contrast induced nephropathy in patients with renal impairment and contrast allergy[59]. It has been suggested that the role of CT angiography in evaluation of patients with acute GI bleeding is in those who are stable and when upper endoscopy or colonoscopy is unable to locate the site of bleeding. Patients with massive GI hemorrhage with hemodynamic instability are recommended to proceed directly to catheter angiography or urgent surgery[38].

Catheter angiography

Catheter angiography can detect bleeding at rates of 0.5 to 1.5 mL/min[60,61]. It is used often in suspected acute lower GI bleeding due to anatomical availability of end arteries and is more challenging in acute upper GI bleeding due to the presence of multiple collateral vessels[62]. In comparison to other imaging modalities it offers the advantages of being both a diagnostic and therapeutic tool allowing for infusion of vasoconstrictive drugs and/or embolization (Figure 3E and F). It also does not require bowel preparation. The sensitivity for a diagnosis of acute GI bleeding is 42%-86% with the specificity close to 100%[63]. Other factors that may affect the sensitivity of angiography include intermittent bleeding, procedural delays, atherosclerotic anatomy, and venous or small vessel bleeding[64,65].

Complications include access-site hematoma or pseudoaneurysm, arterial dissection or spasm, bowel ischemia, and contrast-induced nephropathy or allergic reaction. Complications occur in 0%-10% of patients undergoing angiography, with the incidence of serious complications occurring in < 2% of patients[48,66]. It is recommended that catheter angiography be reserved for patients in whom endoscopy is not feasible due to severe bleeding with hemodynamic instability, or in those with persistent

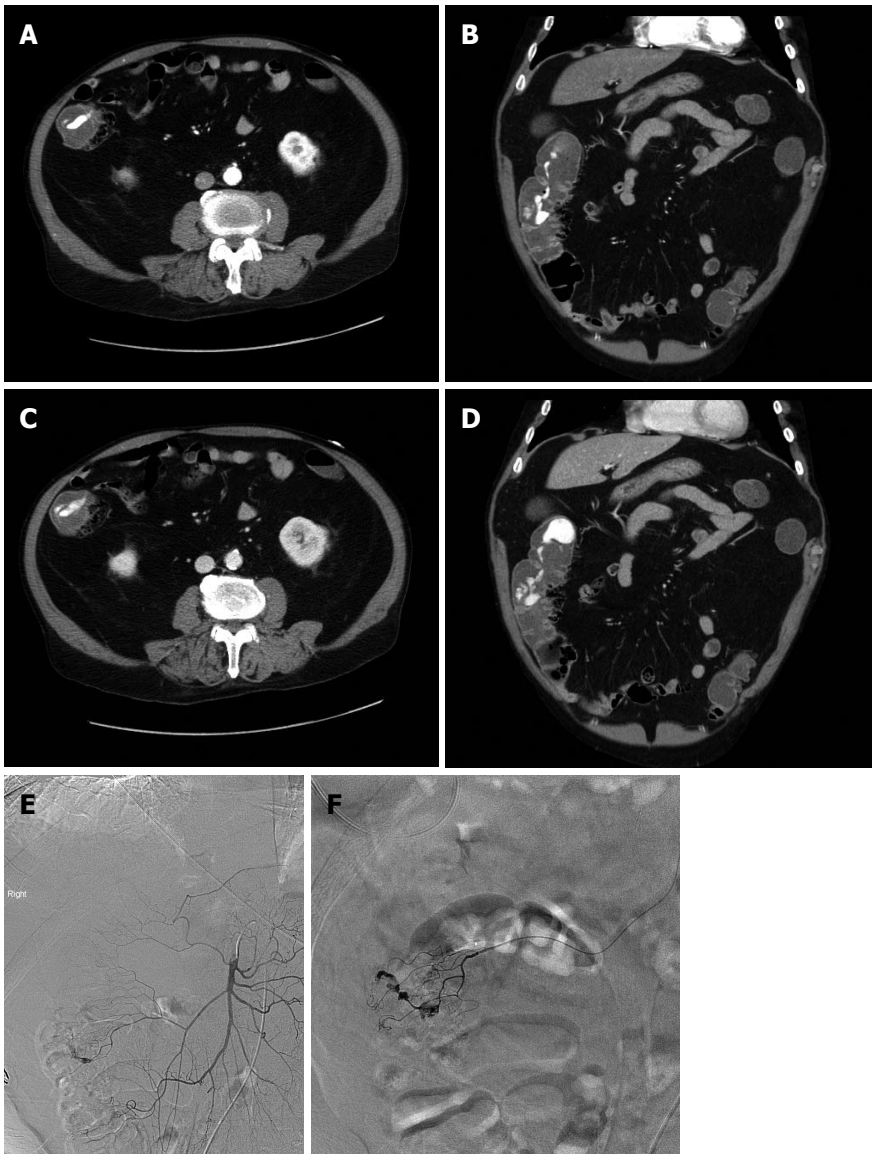

Figure 3 73-year-old man with per rectal bleeding and active gastrointestinal hemorrhage. Contrast enhanced computed tomography (CT) angiogram images show extravasation of contrast into the lumen of the ascending colon, with pooling of contrast which increases from the arterial phase (A, B) to the delayed venous phase (C, D). Diverticula are seen arising from the medial wall of the ascending colon indicating the etiology of bleeding. Following the CT angiogram, the patient underwent catheter angiography, which demonstrated blush of contrast from the right colic branch of the superior mesenteric artery (E). Selective catheterization of the right colic artery demonstrates the bleeding focus more clearly (F). Gelfoam and coil embolization was subsequently performed.

or recurrent GI bleeding and a non-diagnostic upper endoscopy and/or colonoscopy[20].

Radionuclide imaging

The threshold rate of GI bleeding for localization with radionuclide scanning is 0.1 mL/min, and this is the most sensitive imaging modality for GI bleeding[67]. Nuclear scans are either technetium-99m (^{99m}Tc) sulphur colloid or ^{99m}Tc pertechnetate-labelled autologous red blood cells. The short half-life of ^{99m}Tc sulphur colloid is a limitation as this means that patients must be actively bleeding during the few minutes the label is present in the intravascular space, and repeat scanning for intermittent bleeding is not possible without reinjection. ^{99m}Tc pertechnetate-labelled red blood cell scan allows for frequent abdominal images up to 24 h if necessary and is more commonly utilized for investigation of patients with obscure, intermittent bleeding. The main disadvantage of this test is poor anatomic localization of the bleeding site, and this poorly predicts subsequent angiogram results[68,69]. Furthermore, radionuclide only provides functional data, and is unable to diagnose the pathological cause of GI bleeding. Although advocated as a guide for surgical resection, surgical planning should not be based on only a positive nuclear scan[70].

All imaging studies have the advantage of allowing the clinician to identify the location of bleeding throughout the GI tract, especially those originating from the small bowel. However, their use is often limited by the need for active bleeding at the time of investigation. Other diagnostic modalities such as push enteroscopy, deep small bowel enteroscopy and capsule endoscopy may be

of value when the above described investigations prove to be non-diagnostic and when patients are hemodynamically stable with low volume bleeding. These studies will be discussed in the subsequent section evaluating chronic occult GI bleeding.

OCCULT (CHRONIC) GI BLEEDING

Epidemiology

Chronic occult GI bleeding occurs in the setting of a positive FOBT and/or iron deficiency anemia. Iron deficiency is the most common cause of anemia worldwide. In developed countries the major cause of iron deficiency is secondary to chronic blood loss[71]. In the United States, it is estimated that 5%-11% of women and 1%-4% of men are iron deficient and 5% and 2% of adult women and men have iron deficiency anemia, respectively[72]. Iron deficiency anemia has traditionally been attributed to chronic occult GI bleeding, especially in groups other than premenopausal women, and warrants further investigation of the gastrointestinal tract, including for colorectal cancer[12].

Etiology and pathophysiology

Chronic occult GI bleeding may occur anywhere in the GI tract, from the oral cavity to the anorectum[73]. In a systematic review of five prospective studies, 29%-56% of patients had an upper GI source and 20%-30% of patients had a colorectal source of occult GI bleeding diagnosed by the means of upper endoscopy and colonoscopy. These studies were unable to identify a source in 29%-52% of patients[74]. Causes of chronic occult GI bleeding can be broadly categorized into mass lesions, inflammatory, vascular, and infectious[12]. More common causes include colorectal cancer (especially right-sided colon), severe esophagitis, gastric or duodenal ulcers including from the use of aspirin and other NSAIDs, inflammatory bowel disease, gastric cancer, celiac disease, vascular ectasias (any site), diverticula, and portal hypertensive gastropathy. Non-GI sources of blood loss such as hemoptysis and oropharyngeal bleeding can also cause a positive FOBT[75]. A small bowel source accounts for a high percentage of patients with chronic occult GI bleeding and negative findings on upper endoscopy and colonoscopy[10], which is classified as obscure GI bleeding.

Clinical presentation

Patients with iron deficiency anemia may or may not be symptomatic. Rockey[75] recommended that initial investigation be directed towards the location of specific symptoms if possible. In the absence of symptoms, particularly in the elderly, the colon should be evaluated first, and if this is negative, upper GI tract is further investigated[75]. A targeted history is of value to discern symptoms of unintentional weight loss (suggestive of malignancy), use of aspirin or other NSAIDs (ulcerative mucosal injury), antiplatelet or anticoagulant use, family history, liver disease, and previous gastrointestinal tract surgery[76]. Physical signs could indicate presence of an underlying condition such as celiac disease, inflammatory bowel disease, Plummer-Vinson syndrome, and Peutz-Jeghers syndrome[74].

Investigations

Once a patient has been identified as having positive FOBT and/or iron deficiency anemia, multiple diagnostic procedures are available for investigation of the GI tract. The choice and sequence of procedures will depend on clinical suspicion and symptoms[10]. Endoscopic measures include upper endoscopy, colonoscopy, deep enteroscopy, or capsule endoscopy. CT colonography, CT and magnetic resonance (MR) enterography are some of the radiographic investigations utilized in the evaluation of patients with chronic occult GI bleeding. The role of barium enema, small bowel series, enteroclysis, standard CT or MR imaging and nuclear scans have substantially declined due to their low diagnostic yield and the advent of capsule endoscopy[11]. The choice of investigation should also incorporate consideration of patient risk factors and preference. In general, colonoscopy and upper endoscopy are the initial investigations of choice for chronic occult GI bleeding[11].

Colonoscopy and upper endoscopy

The 2007 American Gastroenterological Association guidelines on obscure GI bleeding recommended that the evaluation of a patient with a positive FOBT depends upon whether iron deficiency anemia is present. Patients with positive FOBT and no anemia should first be investigated with a colonoscopy (if upper GI symptoms present then also upper endoscopy) whereas patients with iron deficiency anemia should undergo both upper endoscopy and colonoscopy[11]. Patients with negative findings on upper endoscopy and colonoscopy without anemia do not require further investigations, but those with anemia should be referred for further investigation of the small bowel. The initial small bowel investigation of choice, when available, is wireless capsule endoscopy[11].

Capsule endoscopy, push enteroscopy and deep enteroscopy

Wireless capsule endoscopy is a simple, non-invasive method to study the small intestine for evaluation of small intestinal occult GI bleeding (Figure 4). The diagnostic yield in patients with chronic occult and obscure GI bleeding (after negative upper endoscopy and colonoscopy) ranges from 55%-92% for capsule endoscopy[77,78] in comparison to 25%-30% for push endoscopy[79,80]. A meta-analysis of 14 studies demonstrated that the diagnostic yield of capsule endoscopy was superior to push enteroscopy (63% *vs* 28%) and barium studies (42% *vs* 6%)[81]. Capsule endoscopy also avoids the higher rates of morbidity and mortality associated with push enteroscopy[82]. Capsule endoscopy is less useful in evaluating colonic sources of bleeding because of retained stool, battery life and poor field of vision due

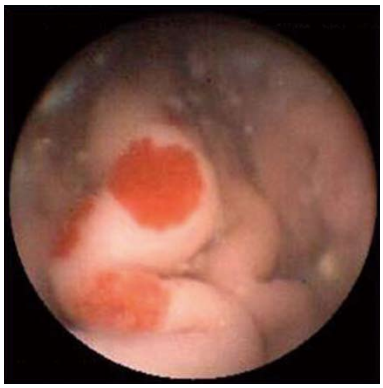

Figure 4 Jejunal angiodysplasia as seen on capsule endoscopy.

to the colon's large diameter[48]. Complications related to the procedure are rare and include capsule retention and obstruction[83].

Push enteroscopy can evaluate the GI tract to 60-80 cm of the proximal jejunum. However, with the availability of deep enteroscopy, which can reach to the distal small bowel, the use of push enteroscopy has diminished. Three systems widely used are: the double balloon endoscopy system, the single balloon enteroscope system, and the Endo-Ease Discovery SB small bowel enteroscope or spiral enteroscope, and may be performed *via* the oral or anal route[10]. Studies comparing the three different modalities are lacking. The advantage of deep enteroscopy over capsule endoscopy is that it can also be a therapeutic modality. The diagnostic yield of double-balloon enteroscopy varies from 40%-80% and therapeutic success ranging between 15%-55%[84,85].

Radiographic imaging modalities

Historically, an upper GI series with small bowel follow-through and/or enteroclysis was the next test performed, but in recent years, where available, CT and MR enterography have superseded these older radiographic modalities.

CT enterography involves ingestion of a neutral contrast agent to distend the small bowel which enables better evaluation of the small bowel wall in comparison to barium solutions. The alternative is MR enterography which has the advantage of not using ionizing radiation allowing serial imaging of the small bowel.

Compared to capsule endoscopy, CT enterography provides better visualization of the entire small bowel wall and shows extra-enteric complications of small bowel disease, whereas capsule endoscopy allows direct visualization of the small bowel mucosa and has a higher sensitivity for mucosal processes[86].

OBSCURE GI BLEEDING

Obscure GI bleeding accounts for 5% of patients of all cases of GI bleeding, both acute overt and chronic occult[12,76]. It is defined as recurrent bleeding when the source remains unidentified after endoscopic procedures and is most commonly caused by bleeding from the small intestine. The commonest causes of obscure GI bleeding include small bowel tumors, vascular anomalies such as angiodysplasias and varices, diverticula and Celiac disease. The emphasis in diagnosis of obscure GI bleeding is the investigation of the small bowel[76].

Repeat upper endoscopy and/or colonoscopy should be considered as one study using double-balloon enteroscopy showed that 24.3% of obscure GI bleed were of non-small bowel origin and within the reach of conventional upper and lower endoscopes[87]. The already mentioned small bowel investigations using capsule endoscopy and deep enteroscopy techniques (including double-balloon enteroscopy, single-balloon enteroscopy and spiral enteroscopy) have enabled the diagnosis of substantially more cases of obscure GI bleeding. Independent series showed that capsule endoscopy had a diagnostic yield of 53%-68% in obscure GI bleeding, led to a specific intervention in the majority of patients and was associated with a significant reductions in hospitalizations and blood transfusions[88,89]. In a randomized controlled trial in patients with iron deficiency anemia and obscure GI bleeding, capsule endoscopy identified a bleeding source significantly more than push enteroscopy (50% *vs* 24%, $P = 0.02$)[90]. Double-balloon enteroscopy was shown in a systematic review to have a diagnostic yield of approximately 68% in obscure GI bleeding[91]. A meta-analysis of studies comparing capsule endoscopy and double-balloon enteroscopy concluded comparable diagnostic yield (60% *vs* 57%, $P = 0.42$) in small bowel disease and obscure GI bleeding[92]. Capsule endoscopy has the major advantage of being less invasive than deep enteroscopy but the major advantage of deep enteroscopy techniques is their ability to perform treatment at the same time. The choice between capsule endoscopy and deep enteroscopy should be individualized for each patient and one approach may be initial capsule endoscopy followed by a directed deep enteroscopy as directed intervention[76].

CT or MR enterography may be considered as an alternative investigation for small bowel disease due to its ability to visualize the small bowel wall and extra-enteric complications, especially when capsule endoscopy and deep enteroscopy are non-diagnostic. In patients with signs of active bleeding, the above mentioned technetium-99 radionuclide scan, CT angiography and catheter angiography should be considered to help locate the lesion prior to intervention.

CONCLUSION

GI bleeding can be caused by a wide range of pathologies and they differ in onset, location, risk and clinical presentation. In patients with active GI bleeding who are unstable, acute resuscitation should precede any investigations. Accurate clinical diagnosis is crucial in determining the investigation of choice and specific treatment interventions. The correct diagnostic algorithm (Figure 5) relies on a good understanding of the type of GI bleeding, risk

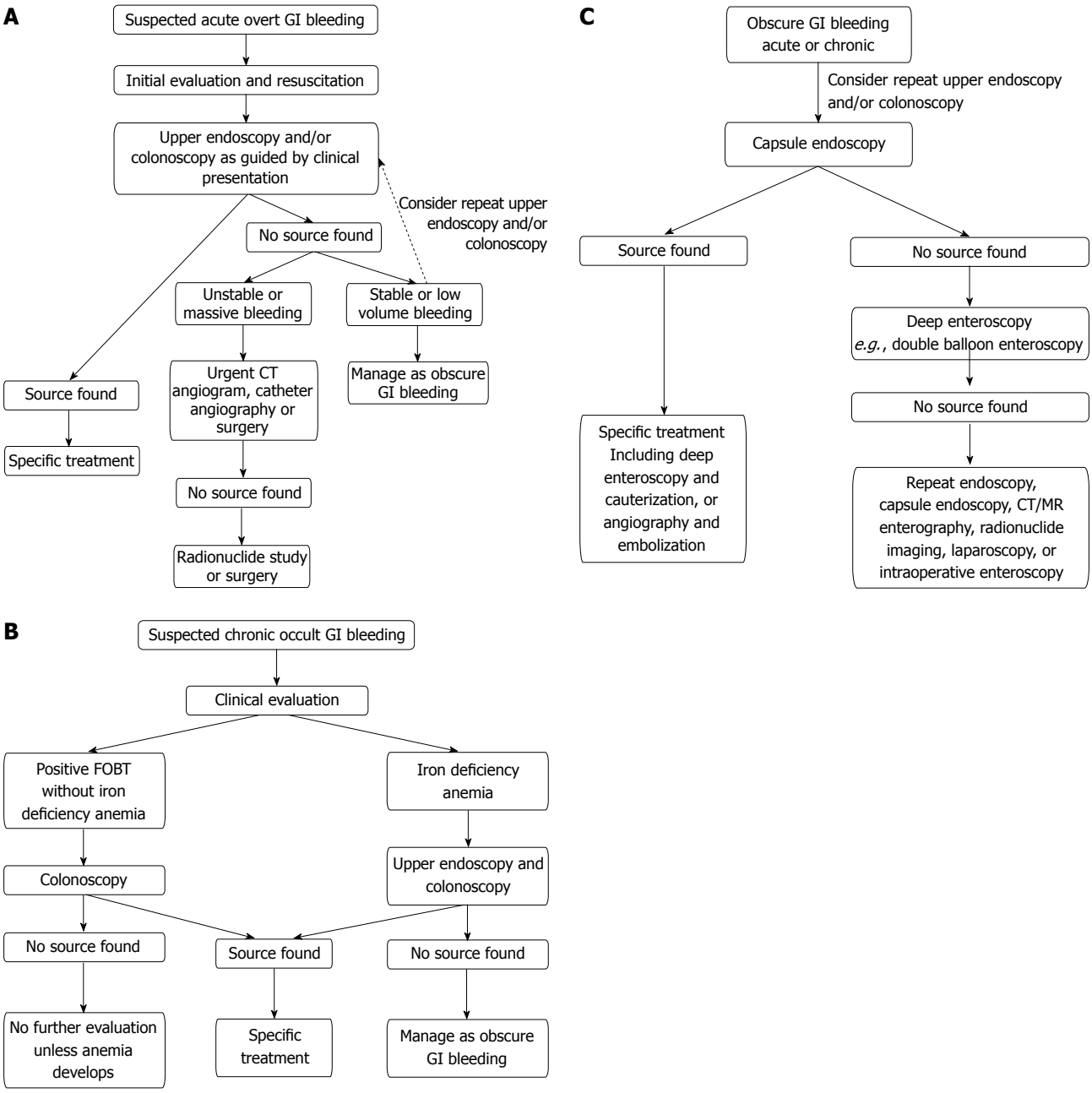

Figure 5 Diagnostic algorithms. A: Acute overt; B: Chronic occult; C: Obscure. CT: Computed tomography; MR: Magnetic resonance; GI: Gastrointestinal; FOBT; Fecal occult blood test.

evaluation and clinical presentation which may indicate the nature and source of bleeding. Upper endoscopy and colonoscopy are the mainstay of initial investigations. Angiography and radionuclide imaging are best suited for acute overt GI bleeding. Capsule endoscopy and deep enteroscopy play significant roles in the diagnosis of obscure GI bleeding, usually from the small bowel.

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TOPIC HIGHLIGHT

WJGP 5th Anniversary Special Issues (9): Gastrointestinal bleeding

Evaluation and outcomes of patients with obscure gastrointestinal bleeding

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Abstract

Obscure gastrointestinal bleeding (OGIB) is defined as recurrent or persistent bleeding or presence of iron deficiency anaemia after evaluation with a negative bidirectional endoscopy. OGIB accounts for 5% of gastrointestinal bleeding and presents a diagnostic challenge. Current modalities available for the investigation of OGIB include capsule endoscopy, balloon assisted enteroscopy, spiral enteroscopy and computed tomography enterography. These modalities overcome the limitations of previous techniques. Following a negative bidirectional endoscopy, capsule endoscopy and double balloon enteroscopy remain the cornerstone of investigation in OGIB given their high diagnostic yield. Long-term outcome data in patients with OGIB is limited, but is most promising for capsule endoscopy. This article reviews the current literature and provides an overview of the clinical evaluation of patients with OGIB, available diagnostic and therapeutic modalities and long-term clinical outcomes.

Key words: Obscure gastrointestinal bleeding; Capsule endoscopy; Double balloon enteroscopy; Outcomes; Anaemia

Core tip: This article examines the role of current diagnostic modalities for the investigation of obscure gastrointestinal bleeding (OGIB) and outcomes in patients undergoing these investigations. Capsule endoscopy and double balloon enteroscopy remain the cornerstone of diagnostic and therapeutic management. The diagnostic and therapeutic capabilities of certain modalities are influenced by the nature of bleeding in OGIB. Long-term outcome data in patients with OGIB is limited but is most promising for capsule endoscopy.

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INTRODUCTION

Obscure gastrointestinal bleeding (OGIB) is defined as recurrent or persistent bleeding or presence of iron deficiency anaemia (IDA) after negative evaluation with oesophagogastroduodenoscopy (OGD) and colonoscopy[1]. OGIB can be categorised further into overt or occult obscure gastrointestinal (GI) bleeding. Overt GI bleeding refers to patients with clinically evident bleeding (haematemesis, melaena or haematochezia) whereas occult GI bleeding occurs in the setting of persistent IDA or a positive faecal occult blood test.

OGIB accounts for approximately 5% of GI bleeding. In more than 80% of cases, the bleeding arises from the small bowel distal to the Ampulla of Vater and proximal to the ileocaecal valve rendering it relatively inaccessible to traditional endoscopy[2-4]. Patients with OGIB

Table 1 Aetiology of obscure gastrointestinal bleeding

Vascular	Inflammatory	Neoplastic	Extraluminal	Rare causes
Angioectasias	Inflammatory bowel disease	Carcinoid	Haemobilia	Hereditary Haemorrhagic Telangiectasias
Dieulafoy's Lesion	Peptic ulcer disease	Gastrointestinal stromal tumour	Aortoenteric fistula	Von Willebrand disease
Gastric antral vascular ectasia	Oesophagitis	Adenocarcinoma	Haemosuccus pancreaticus	Amyloidosis
Portal hypertensive gastropathy	Cameron erosions	Metastases (melanoma)		Henoch Schonlein Purpura
Varices	Meckel's diverticulum	Lymphoma		
Radiation enteritis	NSAID related gastropathy/ enteropathy	Ampullary carcinoma		
Haemorrhoids				

NSAID: Non-steroidal anti-inflammatory drugs.

undergo more investigations, have longer duration of hospitalisation, require more blood transfusions and generate higher healthcare expenditures than patients with upper or lower gastrointestinal bleeding[1]. This is largely due to difficulty accessing the small bowel endoscopically which presents a diagnostic challenge[4].

Current modalities to investigate for OGIB include both endoscopic and radiological techniques. The role of radiological modalities in the evaluation of OGIB has declined substantially as a result of their low diagnostic yield[2]. In this article, we review the clinical evaluation and outcomes of patients presenting with OGIB.

EVALUATION OF OGIB

The clinical history may suggest the possible cause and location of OGIB but it is rarely diagnostic. Endoscopic evaluation remains the cornerstone of diagnosis and management in OGIB[5]. A careful history is key and should include the nature (occult or overt) and clinical presentation of GI bleeding (haematemesis, melaena, haematochezia). Further history regarding other gastrointestinal symptoms (weight loss, obstructive symptoms), medications (anticoagulants, non-steroidal anti-inflammatory drugs), comorbidities (haematological disease, valvular heart disease), prior surgeries (abdominal aortic aneurysm repair, bowel surgery), and family history (inflammatory bowel disease, malignancies, familial telangiectasias) may give clues to the underlying cause[6]. While haematemesis reliably localises the bleeding proximal to the ligament of Treitz, stool colour is a less reliable indicator as it is dependent upon intestinal transit time. Elderly patients, patients with valvular heart disease, renal disease or connective tissue disease are at high risk of vascular lesions. Use of non-steroidal anti-inflammatory drugs (NSAIDs) increases the risk of small bowel ulceration[7]. Physical examination may be useful in detecting systemic syndromes such as hereditary haemorrhagic telangiectasias or Coeliac disease[6].

The most common causes of OGIB vary according to age (Table 1). In patients younger than 40 years of age, small intestinal tumours, Crohn's disease, Meckel's diverticulum, polyposis syndromes and angiodysplasias predominate, whereas patients older than 40 years of age are more likely to bleed from vascular causes (*e.g.,* angiodysplasias) and NSAID enteropathy[8,9]. Causes of OGIB are mainly vascular in the Western population and ulcerations or erosions in the Asian population[10]. Patients who present with IDA without gastrointestinal symptoms, may have gastrointestinal diseases that cause iron malabsorption such as Coeliac disease, atrophic gastritis and Helicobacter Pylori gastritis[11].

Availability of procedures, patient preferences, physician expertise, costs and risks are important determinants of investigation and management[12].

CAPSULE ENDOSCOPY

Capsule endoscopy (CE) has revolutionised the ability to image the small bowel. It is commonly used as a first-line diagnostic tool for investigation of OGIB.[1] This is due to its non-invasiveness, patient tolerance, high negative predictive value (80%-100%) and high diagnostic yield[3,13,14]. CE enables direct visualisation of the small bowel mucosa and has a high sensitivity for detecting flat lesions, such as angiodysplasias, ulcers and arteriovenous malformations which are not easily detectable on radiological modalities[15].

The reported diagnostic yield in literature ranges from 58.4% to 86.8%[9,14,16-21]. The wide range is attributable to different definitions of a positive finding on CE. The diagnostic yield is not affected by age, rendering it a useful test across all age groups[22]. However, it is affected by patient factors including ongoing bleeding, low haemoglobin and ongoing transfusion requirements[23].

Pennazio *et al*[16] reported that the diagnostic yield of CE was significantly higher in patients with ongoing overt OGIB (92.3%), intermediate in patients with occult OGIB (44.2%) and lowest in patients with previous overt OGIB (12.9%). In the overt OGIB group, the diagnostic yield was inversely proportional to the length of time since the last bleeding episode, as delay in the use of CE allows for healing of the bleeding site[24]. CE thus has its highest diagnostic yield in patients with ongoing and overt bleeding[16,25].

CE has been shown to be superior to other modalities

including computed tomography, and small bowel barium studies[26-29]. When compared to push enteroscopy (PE), two meta-analyses have confirmed the superiority of CE, one of which demonstrated a diagnostic yield 30% higher than PE[29,30].

When comparing CE with double balloon enteroscopy (DBE), the literature is inconsistent due to small sample sizes[6]. Teshima *et al*[31]'s meta-analysis comparing CE and DBE in OGIB revealed a similar diagnostic yield (62% *vs* 56%), a finding supported by 2 other meta-analyses[31-33]. CE has a higher diagnostic yield than either anterograde or retrograde DBE alone (OR = 1.61, 95%CI: 1.07-2.43) but not when both approaches are used together (OR = 0.12, 95%CI: 0.03-0.52). This highlights the importance of a total enteroscopy in patients with a high clinical suspicion of small bowel pathology[33]. However, the completion rate of DBE is highly variable (16%-86%)[34,35].

CE has other distinct advantages since it allows the patient to remain ambulatory and requires minimal preparation without sedation[36]. Its main limitation is that it is solely a diagnostic tool lacking therapeutic capacity and the ability to obtain histology[37,38]. It has limited effectiveness in detecting small bowel submucosal tumours, with a false-negative rate up to 19%[39,40]. Other limitations include the inability to precisely locate the bleeding lesions and a small (but significant) risk of capsule retention (0.75% to 5.8%)[3,41,42].

ENTEROSCOPY

PE

PE can visualise the proximal small bowel up to 100cm distal to the ligament of Trietz[6]. It has diagnostic and therapeutic (biopsy, electrocautery, injection, polypectomy) capabilities[4]. An important advantage of PE is that it facilitates a second look for missed lesions within reach of an OGD which is seen in 25%-40% of cases[43,44].

The reported diagnostic yield is between 3%-70%[2,45-47]. The main limitation is its inability to reach lesions beyond the middle jejunum, patient discomfort and its time-consuming nature[4,48]. Complications are rare and include pancreatitis and mucosal injuries[43]. It has largely been replaced by CE for diagnosis and DBE for small bowel endoscopic treatment. Its role mainly lies in the treatment of proximal small bowel lesions found on CE[6].

Double balloon enteroscopy

Double balloon enteroscopy facilitates examination of the entire small bowel[4]. It is considered the gold standard for therapeutic intervention of many small bowel disorders in OGIB[49]. The diagnostic yield and treatment success of DBE for OGIB in published literature ranges from 60%-81% and 43%-84% respectively[10,50-60]. The variation in diagnostic yield is a result of differences in DBE timing, inclusion criteria and definitions of a significant finding[61]. Like CE, DBE has a higher diagnostic yield in patients with overt-ongoing OGIB than overt previous and occult OGIB, suggesting that the time interval between the last bleeding episode and the DBE examination is a key factor in diagnosing the causative lesion in OGIB[10].

The approach of a targeted DBE (after a prior CE) has been shown to increase both its diagnostic (73%-93%) and therapeutic yield (53%-73%)[38,62,63]. DBE can change or improve the diagnosis in a significant number of patients in whom CE is performed beforehand. In a study by Kaffes *et al*[38], DBE after CE clarified or made a new diagnosis in 20% of patients. A CE guided DBE is likely to diminish the need for total enteroscopy in most patients, as demonstrated by Gay *et al*[62] who showed a high positive predictive value for CE to correctly predict the DBE approach. The targeted approach is also useful in confirming indeterminate findings from CE. Hence, it is strongly suggested that CE is the initial screening modality in OGIB and that these two investigations should be viewed as complementary[20,64].

Not surprisingly, when compared with PE, a controlled prospective trial on patients with suspected small bowel bleeding, confirmed that anterograde DBE is significantly superior to PE in regards to the detection of pathological lesions (63% *vs* 44%) and the length of small bowel visualised (230 cm *vs* 80 cm)[65].

DBE is restricted by its limited availability, prolonged procedural times and sedation requirements[37]. The complication rate is 0.8% for diagnostic procedures and up to 4% for therapeutics such as polypectomy, electrocautery or dilatation[6]. Complications include bleeding, ileus, intestinal perforation, pancreatitis or those related to sedation[49]. For these reasons, DBE is a second-line investigation in OGIB, reserved for patients with a positive CE who require therapeutic intervention or biopsy[2].

Current guidelines recommend CE as the preferred initial modality in OGIB given its diagnostic yield, outcome data, safety and non-invasive nature. DBE should be viewed as a complementary procedure. It plays an important therapeutic role following diagnostic CE and diagnostic role following negative CE in patients with ongoing bleeding or high suspicion of small bowel pathology. Other scenarios for initial use of DBE are where CE is not available or affordable and in patients with overt OGIB who may benefit from early DBE[64]. More prospective randomised controlled clinical studies are required to determine the most efficient and cost effective use of CE and DBE[61].

Spiral enteroscopy

Spiral enteroscopy utilises a spiral shaped overtube with a raised helix at the distal end. It allows for advancement and withdrawal of the enteroscope through the small bowel by using clockwise and anticlockwise movements respectively[6]. It offers the same diagnostic and therapeutic capabilities as DBE. Initial studies comparing DBE and spiral enteroscopy have suggested that the two procedures have similar diagnostic yields[66-68]. Further studies

comparing spiral enteroscopy to other modalities such as CE and DBE are required.

Intraoperative enteroscopy

Intraoperative enteroscopy (IOE) was previously considered the gold standard of small intestinal imaging. It has the highest sensitivity in detecting bleeding small bowel lesions with a diagnostic yield of 80%-100%[69,70]. This is at the expense of extreme invasiveness making this modality a last resort in the investigation OGIB[4]. Indications of IOE include when small bowel lesions cannot be managed by angiographic embolisation or endoscopic treatment or when surgery is required[70].

REPEAT UPPER AND LOWER ENDOSCOPY

Bleeding sources within reach of upper and lower endoscopy may be missed as a result of small size, atypical location, inadequate endoscopy investigation, slow or intermittent bleeding, or compromised visualisation (due to presence of blood or poor colonic preparation)[6].

Numerous studies demonstrate that a significant proportion of patients with negative initial investigations have a bleeding source on repeat OGD in 35%-75% or repeat colonoscopy in 6% of cases[45,71-76]. Thus a re-look endoscopy may be recommended as a cost-effective first step before further evaluation[7]. Factors associated with increased yield on repeat OGD include large hiatus hernias, history of NSAID use, and haematemesis[45].

Common missed lesions include colonic angiodysplasias, peptic ulcers, Cameron's lesions, gastric antral vascular ectasia and radiation proctitis[49].

The American Gastroenterological Association recommend repeating OGD and colonoscopy if there is suspicion of an overlooked lesion before proceeding to CE or DBE[2]. Repeat OGD and/or colonoscopy should also be considered if suboptimal equipment was used or in the setting of inadequate mucosal visualisation secondary to poor bowel preparation[49].

COMPUTED TOMOGRAPHY ENTEROGRAPHY

Computed tomography enterography (CTE) is a readily available, non-invasive, operator independent method for visualising the small bowel. It can detect extraluminal pathology which is not possible with CE. The overall sensitivity of CTE is low (50%), however it is effective for detecting small bowel tumours (sensitivity exceeding 90%)[77-79]. The diagnostic yield of CE following negative CTE is high, 57% in one study[25]. Small bowel ulcers are the most commonly missed lesions with CTE which are readily detected by CE[1]. However, in patients less than 40 years of age where small bowel tumours are the most common cause of OGIB, CTE should be strongly considered given the aforementioned false negative rate of CE for detecting small bowel neoplasms[80,81].

OUTCOMES

Capsule endoscopy

Although many studies demonstrate a high diagnostic yield of CE for detecting a cause of OGIB, its impact on patient outcomes is more important[82]. With regards to rebleeding rates, Endo *et al*[18] found that among patients with significant CE findings, the rebleeding rate at a mean of 11.6 mo follow up of the patients who underwent therapeutic intervention was significantly lower than that of those without intervention (9.5% *vs* 40.0%, $P = 0.046$). This is supported by other studies[83,84]. Hence, aggressive intervention of patients with significant CE findings reduces risk of rebleeding. Patients with insignificant findings (erosions, small ulcers, red spots, small polyps) or a negative CE, had a significantly higher rate of re-bleeding than those with significant findings on CE. These patients should have careful follow up, whilst being mindful that the bleeding may not be originating from the small bowel[18]. Viazis *et al*[85] found that 65% of patients with a negative initial CE continued to have OGIB after a mean follow up period of 24 mo. Development of overt bleeding and a haemoglobin drop of 4 g/dL or more were significant predictive factors for a diagnostic repeat CE. Similar to its influence on diagnostic yield, the nature of bleeding in OGIB also impacts on rebleeding rates. In the Pennazio *et al*[16] study, complete resolution of bleeding occurred significantly more often in patients with ongoing overt and occult OGIB than with previous OGIB.

In regards to other outcome measures, Leighton *et al*[36] demonstrated significant reductions in the requirement for blood transfusions, gastrointestinal procedures and hospitalisation as well as significant improvements in haemoglobin levels at 1 year follow-up of 20 patients undergoing CE for investigation of OGIB. Hindryckx *et al*[86] also confirmed favourable outcomes in 66.3% of their patients after CE guided therapy which led to a decrease in the need for blood transfusions and significantly higher haemoglobin levels after a mean follow up of 635.5 d.

DBE

Kaffes *et al*[38] reported significant reductions in further bleeding (80%), blood transfusions and iron requirements in a prospective cohort study of 60 patients with positive CE findings undergoing DBE treatment after 10 ± 5.2 mo follow up. Seventy-seven percent of patients maintained a normal haemoglobin. Hsu *et al*[59] similarly found significantly less rebleeding in patients who were treated for an identified lesion when compared to patients in whom no lesion was found (20% *vs* 80%).

Byeon *et al*[87] found that repeat DBE in the same direction may detect a source of bleeding in 53% of recurrent OGIB patients, particularly in patients with a previous positive DBE (81% yield). Angiodysplasias were the most common cause of OGIB in both DBEs. An-

giodysplasia has been identified as a common source of rebleeding in studies exploring outcome in patients with OGIB after PE, CE and or DBE[88,89].

Most studies follow up patients for up to 12 mo. Larger prospective studies with longer follow up are required to evaluate long term outcomes of OGIB patients following DBE.

Push enteroscopy

Several small studies suggest that patient outcomes are improved after PE[4]. In one study of 105 patients with OGIB with a mean follow up of 29 mo, resolution of bleeding occurred in 69% of patients[90]. PE impacts upon clinical management in 40%-50% of patients with OGIB[74,91]. Decreased transfusion requirements and improvement in functional status one year post treatment have been found with PE[92].

Other modalities

There are limited data on outcomes of OGIB patients after investigation with other modalities. However, similar to data from CE, DBE and PE, patients who underwent endoscopic treatment for an identified lesion had better outcomes than those without treatment.

Williamson *et al*[37] followed up 61 patients undergoing spiral enteroscopy for OGIB. The mean time to recurrent overt bleeding was 10.4 months. Patients who had endoscopic treatment for bleeding lesions during spiral enteroscopy were significantly less likely to have further overt bleeding (26% *vs* 64%). Increased haemoglobin levels and reduced requirements for blood transfusions, iron supplementation and additional procedures were all observed after spiral enteroscopy.

A retrospective study of IOE demonstrated, at 32 mo follow up, bleeding had resolved in 52% of patients with OGIB in whom a lesion was detected and treated during IOE. Bleeding persisted in 20% and recurred in 8% of patients[93]. Angiodysplasias were responsible for the majority of patients with ongoing bleeding[4].

In a retrospective study, Shin *et al*[1] showed that CTE discovered the source of bleeding in only 26.7% of patients with OGIB. The overall re-bleeding rate was 21.7% during a mean follow up of 17.6 mo. Again, patients with positive CTE who were treated endoscopically had significantly reduced rebleeding rates. A negative CTE did not predict lower long term rebleeding, and thus these patients should be closely observed and have further diagnostic work up (such as with CE or DBE) if there is a high clinical suspicion of small bowel bleeding.

CONCLUSION

Obscure gastrointestinal bleeding is a common problem and remains a diagnostic challenge to gastroenterologists. Various endoscopic, radiological and surgical modalities exist for the investigation of OGIB each with their own advantages, disadvantages and indications in which they should be used. Both CE and DBE remain the cornerstone of investigation and management of OGIB, with other modalities assuming a more selective role. Ultimately patient factors and resource availability determine the modality used. The short-term outcomes of OGIB patients with a treated lesion are good; however rebleeding is common especially in patients where no source of bleeding was found. Further studies are required to evaluate long-term outcomes. With ongoing development and experience in new techniques, the clinical conundrum that is OGIB may no longer be so obscure.

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REVIEW

Contemporary surgical management of rectovaginal fistula in Crohn's disease

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Abstract

Rectovaginal fistula is a disastrous complication of Crohn's disease (CD) that is exceedingly difficult to treat. It is a disabling condition that negatively impacts a women's quality of life. Successful management is possible only after accurate and complete assessment of the entire gastrointestinal tract has been performed. Current treatment algorithms range from observation to medical management to the need for surgical intervention. A wide variety of success rates have been reported for all management options. The choice of surgical repair methods depends on various fistula and patient characteristics. Before treatment is undertaken, establishing reasonable goals and expectations of therapy is essential for both the patient and surgeon. This article aims to highlight the various surgical techniques and their outcomes for repair of CD associated rectovaginal fistula.

Key words: Rectovaginal fistula; Crohn's disease; Fistula; Advancement flap; Sleeve advancement; Episioproctotomy

Core tip: Rectovaginal fistula secondary to Crohn's disease is a devastating and disabling condition with a significant negative impact on quality of life. Furthermore, these fistulae pose an extremely challenging dilemma for the clinician with unique and often frustrating management challenges. Medical management is often futile and surgery may offer the only chance for cure. In this article, we aim to review the various treatment options to close these difficult to treat fistulae, with an emphasis on surgical technique and complex decision making.

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INTRODUCTION

Fistula-in-ano is the most common perianal manifestation of Crohn's disease (CD) and was first reported by Gabriel[1] in 1921, nine years before Crohn *et al*[2] identified regional enteritis as a clinical entity. These fistulae are classified by their relationship to the sphincter complex as either high (supra- or extra-sphincteric *vs* low (inter- or trans-sphincteric). Low fistulae that transverse the anal sphincter are more appropriately named anovaginal fistulae, but by convention, all such fistulae are termed rectovaginal fistula (RVF). After obstetrical trauma, CD is the most common etiological factor for RVF, and will occur in up to 10% of women with CD[3,4].

Rectovaginal fistulae secondary to CD are associated with significant morbidity and carry an increased risk for proctectomy[5,6]. It is a devastating and disabling condition and is a source of considerable social embarrassment

and has a significant negative impact on quality of life. Furthermore, CD associated RVF are an extremely challenging dilemma for the clinician and present unique and often frustrating management challenges. In this article, we aim to review the various treatment options to close these difficult to treat fistulae, with an emphasis on surgical technique and decision making.

PRESENTATION AND DIAGNOSIS

The presence of a fistulous tract between the gastrointestinal tract and the vagina can be distressing and embarrassing for the patient. The most common symptoms includes passage of either gas and/or stool *via* the vagina. Women may also report purulence from the vagina, dyspareunia, perineal pain and tenderness, along with vaginal irritation and recurrent genitourinary tract infections[3,7]. Physical examination may demonstrate the fistulous opening on inspection of the lower anorectum and vagina, but often, the RVF is not visible on inspection. The clinician must have a high index of suspicion when a women presents with signs and symptoms consistent with a RVF. These patients are best evaluated with an examination under anesthesia for definitive elucidation of the RVF[3].

Several other studies are available to help identify and delineate RVF including computed tomography (CT) scan, magnetic resonance imaging (MRI), fistulography, and endoluminal ultrasound (EUS). The use of EUS with hydrogen peroxide enhancement has been advocated in the evaluation of complex fistula disease to visualize side tracts and areas of fluid collection[3,8-10]. Sloots *et al*[10] reported on this modality in 41 patients with CD related fistula-in-ano (32% with RVF), and found that 78% of the patients had a more complex fistula found during EUS. An added benefit of using EUS to evaluate the fistula tract, is the ability to identify any anal sphincter defects.

After a comprehensive workup and evaluation of the perineum, the remainder of the small and large bowel, rectum, and anal canal must be investigated. It is important to identify any other active areas of CD in order to plan both medical and potential surgical management. Work-up may include a colonoscopy, esophagogastroduodenoscopy, small bowel series, CT or MRI enterography. If proximal CD is found, optimization with medical and/or surgical management should be strongly considered before any attempt to repair the RVF.

TREATMENT OPTIONS

There are several disease characteristics that guide treatment recommendations for patients with RVF. These include the location of the fistula (high, low, or transsphincteric), anal canal disease (ulcerations or stricturing), the presence of active inflammation in the rectum, and rectal compliance. The presence and severity of symptoms, discomfort, and quality of life also weigh heavily in regards to treatment type and timing. Because there is considerable debate with regards to the best treatment options for these notoriously difficult to close fistulae, a frank discussion setting realistic goals and expectations of treatment is the initial step. Patients with no or minimal symptoms may actually be advised to have no treatment at all[7,11-12]. For patients with intolerable symptoms, a logical and stepwise approach to management begins with conservative medical therapy and advances to surgical intervention when indicated[3]. It should be noted that there are currently no prospective, randomized, controlled trials for the surgical correction of CD related RVF. The factors previously discussed along with personal experience, surgical judgment and a critical appraisal of the available literature should be used to formulate an optimal and tailored treatment plan for women with CD associated RVF[3].

MEDICAL MANAGEMENT

Traditionally treatment of CD associated RVF has been mostly surgical as medical treatments were fraught with failure[3]. Medical treatment has centered on pharmacological therapy aimed at alleviating and treating the underlying active CD along with medication to alter stool consistency and control diarrhea. Current medications targeting CD include antibiotics, corticosteroids, immunomodulators, and biologics. Metronidazole has been reported to successfully treat RVF, although most surgeons will use this and other antibiotics as an adjunct to surgical treatment[3,13]. Present *et al*[14-16] have written extensively on various medical modalities for the treatment of all types of CD related fistulae, including, cyclosporine, 6-mercatopurine, and infliximab. A randomized, double-blinded, multicenter study by Present *et al*[16] studied infliximab for the treatment of both abdominal and perianal fistulae from CD. After 18 wk of infliximab treatment the authors found significant reduction in the number of fistulae with complete closure occurring in 46% *vs* 13% of placebo. The follow-up was relatively short (4.5 mo) and the study included all enterocutaneous fistulae, not specifically RVF, making generalizability to RVF somewhat limited.

In the ACCENT II study by Sands *et al*[17], the authors evaluated the effect of infliximab in patients with RVF secondary to CD. Twenty-five patients were enrolled and received infliximab infusions at weeks zero, two, and six. Initial responders (those who showed a 50% reduction in their fistula in the first ten weeks) were then randomized to continue receiving infliximab or placebo. At 54 wk follow-up, 44% of the initial responders healed their fistulae and alternatively 56% had RVF recurrence, regardless of infliximab treatment. Essentially, the women who initially responded to the infliximab had a 50% chance of fully healing their RVF.

It is unclear which RVF will respond to infliximab nor is there evidence that infliximab will reduce fistula recurrence rates. At our institution we tend to recommend infliximab (or other biologic therapy) as initial treatment when surrounding tissues are inflamed or ulcerated such

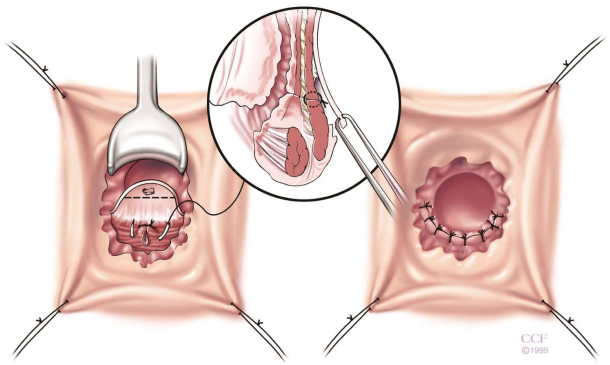

Figure 1 Rectal advancement flap. The rectal advancement flap begins with a 180 degree curvilinear incision starting just distal to fistula opening and extends 4-5 cm cephalad, encompassing mucosa, submucosa and the rectal wall is dissected from the rectovaginal septum. After mobilization, the fistula tract is cored out and the opening is closed with absorbable sutures. The diseased distal portion of the flap is trimmed before and the flap is advanced distally and sutured to the cut edge with absorbable sutures. The vaginal or perineal external opening is left open for drainage. Reprinted with permission, Cleveland Clinic Center for Medical Art and Photography © 1999-2014. All Rights Reserved.

that any attempt at surgical closure will uniformly fail. In some patients, the RVF may close with this therapy, but if it persists and the active inflammation becomes quiescent, then surgical correction may be attempted.

SURGICAL MANAGEMENT

Local repair of RVF secondary to CD can be successfully accomplished when optimal conditions exist. The approaches to local repair include transperineal, transvaginal, and transanal (with or without transabdominal mobilization) techniques. The choice of technique depends on the experience of the surgeon, location of the RVF, and the status and extent of local and distant inflammatory bowel disease activity. Additionally, anal sphincter integrity in women after vaginal delivery must be considered as some patients may require a sphincter repair along with the fistula repair.

An important aspect of RVF repair is initial drainage of perianal sepsis before consideration of surgical closure. Often, the use of loose draining setons is required for adequate sepsis control. The addition of antibiotics may also benefit selected patients with significant purulence. There is a group of women that benefit from a diverting stoma to facilitate sepsis eradication. Typically a stoma is helpful if stool consistency is loose and/or frequent. An added benefit of the stoma procedure is that is allows surgical treatment of intestinal CD at the same operation. Consideration of each of these steps is mandatory before any definitive attempt at RVF repair is undertaken. A waiting period of at least 3-6 mo is needed for all local inflammation and infection to be cleared.

It should be noted that in women with active anorectal disease, the use of a draining seton may be used indefinitely as a sphincter-saving procedure. Seton use in this situation has been shown to successfully preserve fecal continence and delay or avoid a permanent stoma in those women who cannot undergo local surgical repair[3,7,18,19].

Simple fistulotomy

Low and superficial (anovaginal) fistulas can be layed open or excised with a simple fistulotomy in very few select cases with successful healing. These circumstances are rare and virtually no sphincter muscle must be involved for this technique to be considered. If there is any anal canal deformity after fistulotomy (keyhole deformity), some degree of fecal incontinence will undoubtedly result[3,7].

Anocutaneous flap

The anocutaneous flap technique is rarely utilized but may be considered in situations where anal stenosis is present. The technique consists of mobilizing an island of skin and subcutaneous tissue from the anal margin or verge and advancing this flap into the anal canal to cover the RVF. This procedure is only possible if the anal skin is soft and pliable, which is not common in perianal CD patients. Hesterberg *et al*[20] reported a 70% healing rate at median follow-up of 18 mo with this technique.

Transrectal approaches

Most authors believe that repairing RVF from the high pressure (rectal) side of these low pressure fistulas is advantageous. This allows for the source of the fistula to be excised and closed. Then a healthy layer of tissue (flap) is used to cover the repair[7,21,22]. There is a wide variety of flap configurations, but the standard curvilinear rectal advancement flap is the most commonly performed flap procedure for RVF.

Rectal advancement flap

The rectal advancement flap (RAF) repair has been somewhat successful in healing RVF secondary to CD and should be considered in women with favorable anorectal anatomy[3]. Patients with minimally diseased or normal rectum and a normal anal canal are ideal candidates for this type of repair. However, this technique is contraindicated in women with extensive ulceration or stricturing of the anal canal and transitional zone as well as women with an anterior sphincter defect[3]. It should also be used with caution in woman with fecal incontinence. The technique has been well described in the literature, but briefly, it consists of making a curvilinear incision nearly 180 degrees just distal to the fistula opening in the anal canal. The mucosa of the cephalad anal canal is removed and then a flap of mucosa, submucosa and the rectal wall is dissected from the rectovaginal septum cephalad for approximately 4-5 cm. After sufficient mobilization to avoid tension when advancing the flap, that fistula is cored out and the fistula opening closed with absorbable suture. The flap is then trimmed and advanced distally to the cut edge. Then using absorbable sutures it is sewn to this cut edge with deep bites. The vaginal or perineal external opening is left open for drainage (Figure 1).

Hull and Fazio[23] reviewed forty-eight women who had an anovaginal fistula secondary to CD, with 35 undergoing one of 3 types of flap repairs. Twenty-four women underwent RAF with the standard curvilinear

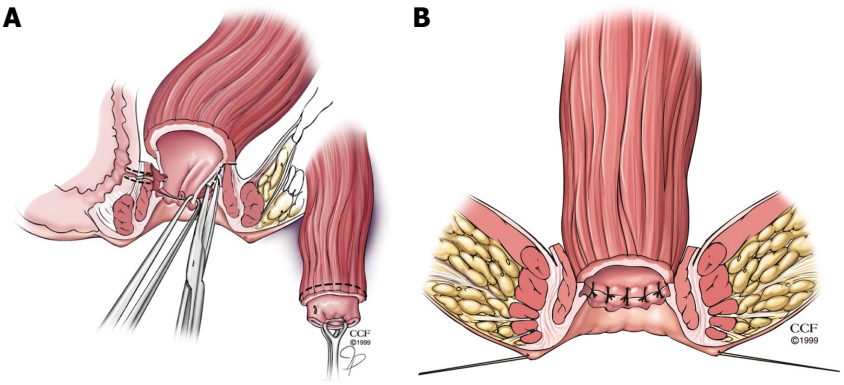

Figure 2 Rectal sleeve advancement flap. A: Dissection begins at the dentate line with a 90%-100% circumferential mucosectomy of ulcerated mucosa and submucosa of the anal canal and is carried cephalad until the supralevator space is breeched. After sufficient rectal mobilization has been accomplished, the fistula tract is cored out and then closed with absorbable suture and the vaginal mucosa is left open; B: The diseased distal margin of tissue is trimmed and the cuff of rectum is advanced down and sutured to the ridge of anoderm using absorbable sutures. Reprinted with permission, Cleveland Clinic Center for Medical Art and Photography © 1999-2014. All Rights Reserved.

incision and six patients with a long and high RVF or the presence of anal ulceration, underwent a linear flap procedure. The initial healing rate of all repair types was 54%, with an ultimate healing rate of 68% after additional surgical procedures were performed. The authors concluded that surgical intervention for low RVF secondary CD is advocated in properly selected patients by using an individualized approach based on the nature of the anovaginal fistula.

In another study by Kodner *et al*[24], endorectal advancement flaps were created in 24 patients with CD and a relatively normal rectum. Seventeen out of twenty-four (71%) patients achieved primary healing after initial flap repair and a total of 22/24 patients had healing after further repairs. Similarly, Makowiec *et al*[25] and Crim *et al*[26] reported successful healing of RVF secondary to CD in 5/12 and 10/14 patients, respectively with this technique.

Ruffolo *et al*[27] stress that the advantages of a flap procedures are a low chance of: producing a keyhole deformity, worsening fecal incontinence, or aggravation of patient's symptoms in case of failure. Additionally, there is no perineal wound and the presence of a stoma is not mandatory.

Rectal sleeve advancement flap

When an endorectal advancement flap is not an acceptable choice for RVF repair due to extensive ulceration or stricturing in the anal canal and transition zone, the rectal sleeve advancement flap may be considered. This technique also requires a normal or near normal rectum. First reported in the literature by Berman in 1991, the rectal sleeve advancement flap removes all of the diseased tissue in the anal canal and allows for a more 'normal' sleeve of rectal tissue to be sutured to the neodentate line[3,28,29]. The rectum should also be distensible and not exhibit any significant scarring from quiescent Crohn's proctitis. Starting at the dentate line, a mucosectomy of the ulcerated mucosa and submucosa of the anal canal is performed. The mobilization is 90%-100% circumferentially. Next the dissection breaches into the supralevator space and the rectal mobilization is continued cephalad until sufficient mobility is achieved so the rectum can be advanced within the sleeve of the internal sphincter to the neodentate line without tension. Anteriorly the dissection is in the rectovaginal septum. The fistula tract is then cored out and sutured, leaving the vaginal mucosa open as was discussed in the RAF (Figure 2A). The diseased distal margin of tissue is trimmed, and the cuff of rectum is advanced down and sutured to the ridge of anoderm, again using absorbable sutures as was described for the RAF (Figure 2B)[23]. In the event that sufficient mobility cannot be obtained to bring the sleeve of tissue to the cut distal edge without tension, the patient and the surgeon must be prepared to convert the operation to a transabdominal approach, with full mobilization of rectum, descending colon, and splenic flexure. Additionally, if a tension -free anastomosis cannot be achieved, proctectomy with end stoma may be necessary so the patient must be appraised of this possibility during the informed consent[3].

In a study from our institution, Marchesa *et al*[29] reviewed 13 patients (12 women) with severe perianal CD (11 with RVF, 1 rectourethral fistula, 1 anal canal ulceration) who underwent sleeve advancement as an alternative to proctectomy. All patients had been previously treated with a rectal advancement flap without success. Eight patients had proximal fecal diversion, with six having concomitant bowel resection with a protective stoma at the same time of sleeve advancement. A 60% success rate was achieved by using the sleeve advancement flap in this carefully selected population of patients. Additionally, Simmang *et al*[30] reported successful healing in two patients with RVF secondary to CD using the sleeve advancement flap.

Patient selection and preparation are keys to achieve a satisfactory outcome with the sleeve advancement flap, therefore careful patient selection is crucial[29]. Fecal diversion is a controversial option with this technique and the majority of patients in Marchesa's study appeared to have improved success rates for RVF closure

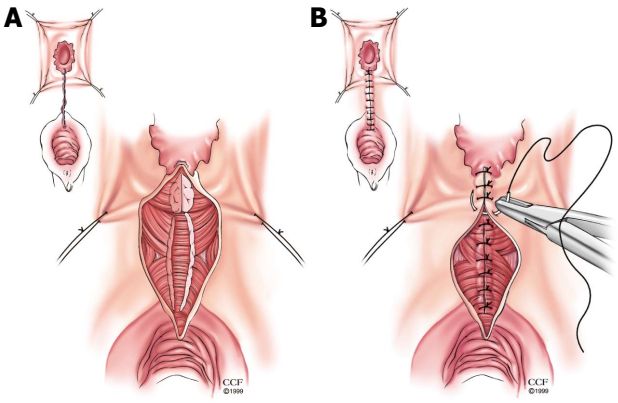

Figure 3 Episioproctotomy. A: Episioproctotomy begins with fistulotomy and division of all tissue overlying the fistula, including sphincter muscles and rectal and vaginal walls. Complete debridement of the granulation tissue of the fistula tract is carried out along with the lateral identification and mobilization of the sphincter muscles; B: The rectal mucosa is repaired followed by an overlap repair of the sphincter muscles. The repair is completed by closing the vaginal mucosa. Reprinted with permission, Cleveland Clinic Center for Medical Art and Photography © 1999-2014. All Rights Reserved.

when they were proximally diverted[29]. It is our practice currently to strongly consider diversion what performing a sleeve. This is typically an ileostomy. Meticulous surgical technique and adherence to principles such as hemostasis, gentle handling of tissues, and debridement of all diseased tissue is of paramount importance in this potentially technically demanding procedure. This repair is typically considered in a patient where the only other alternatives may be total proctocolectomy or permanent fecal diversion[29].

Transperineal approaches

Episioproctotomy: When an anterior sphincter defect coexists in women with an RVF, the surgeon should strongly consider repairing the sphincter defect with the RVF repair[3]. This can be accomplished with either a rectal advancement flap performed in concurrence with an anterior sphincteroplasty[31] or as the case at our institution, an episioproctotomy is performed[32]. An episioproctotomy entails performing a fistulotomy and creating of a defect similar to a fourth degree perineal laceration during vaginal childbirth[32]. A compete debridement of the granulation tissue of the fistula tract is carried out along with lateral identification and mobilization of the sphincter muscles (Figure 3A). The rectal mucosa is repaired initially. Then an overlap of the sphincter muscles is accomplished. Finally the vaginal mucosa is approximated which completes the repair (Figure 3B). El-Gazzaz *et al*[6] from our institution reported their results of various methods of Crohn's related RVF repair. There were 8 women who had an episioproctotomy with a healing rate of 71.4%.

Transverse transperineal repair: Particularly in the gynecological literature, an incision transversely through the perineal body is advocated to repair RVF. Dissection is carried out cephalad to the fistula tract and then the tract is transected with sharp dissection. The posterior vaginal and anterior rectal walls are mobilized and the cicatrix is excised. The vaginal and rectal walls are closed in 2 layers along with a levatoroplasty. Athanasiadis *et al*[33] reviewed various surgical techniques for CD RVF in 37 women undergoing 57 procedures with a mean follow-up of 7.1 years. Twenty women underwent transverse transperineal repair with a 70% overall success rate.

Transvaginal approaches

Vaginal advancement flap: The technique of repairing a RVF *via* the vaginal approach is considered by some surgeons a superior method due to the fact that the operation occurs not in the confines of the anal canal but in the vagina where the tissue is non-diseased, soft and pliable[27]. By avoiding the rectum, there is minimal to no manipulation or instrumentation in the potentially diseased and inflamed bowel. The vaginal advancement flap (VAF) consists of raising a posterior flap of vaginal tissue around the fistula. The rectal and vaginal orifices of the fistula are identified and repaired with absorbable sutures and the levator ani muscle is approximated in the midline. The vaginal flap is then advanced over the repair and sutured to the perineal skin.

Sher *et al*[34] reviewed their experience with 14 VAF for RVF in the setting of CD. They reported 13/14 patients achieved fistula closure. The authors attribute their success with using healthy tissue and also using the levator ani interposition to lend added support and further separation of suture lines. Of note, all 14 patients either had proximal diversion before or at the time of VAF with a loop ileostomy, which the authors felt to be an essential part of their success[34]. Furthermore, in a systematic review of eleven observational studies by Ruffolo *et al*[27], VAF was compared to RAF, with the primary end point of successful RVF closure rate. A total of 219 flap procedures (175 RAF *vs* 49 VAF) were reviewed and the authors noted a 54.2% closure rate after RAF and a 69.4% closure rate for VAF. This review suggests no significant differences in terms of outcome between VAF and RAF in CD. The VAF may be a good surgical option when there is anorectal stenosis or after a failed RVF repair.

Inversion of fistula: If the fistula is low and small, inversion may be an option. A circular incision is made around the vaginal os, and the surrounding flap of vaginal mucosa is mobilized. Several concentric purse-string sutures are placed to invert the fistula into the rectum. The vaginal mucosa is then reapproximated. All surrounding tissue must be soft and pliable for this approach to be considered. It should be noted that there is no reported data on this technique in Crohn's related RVF.

Abdominal approaches

Coloanal anastomosis and turbull-cutait procedure: As mentioned previously, when performing the rectal sleeve advancement, a tension-free anastomosis may not be possible. In this scenario, a transabdominal approach is then used to complete the repair. This can be

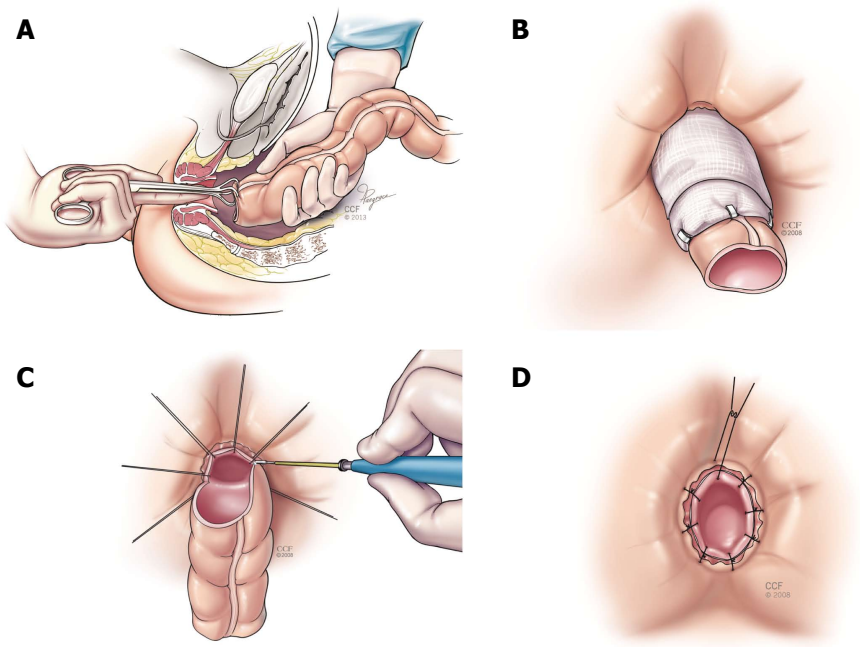

Figure 4 Turnbull-Cutait abdominalperineal pull-through procedure (A-D). Reprinted with permission, Cleveland Clinic Center for Medical Art and Photography © 1999-2014. All Rights Reserved.

done with two main techniques: an immediate hand-sewn coloanal anastomosis or a delayed coloanal anastomosis (Turnbull-Cutait). After complete rectal (and if needed) descending and splenic flexure mobilization, the colon is passed transanally. If the local conditions in the anus are satisfactory, a standard hand-sewn coloanal anastomosis is performed immediately. When there are other fistulous tracts close to the neodentate line or the internal opening of the fistula is close to the suture line, a delayed coloanal anastomosis as described by Cutait and Turnbull[35-37] should be considered. The highlight of this procedure relies on placing 8 sutures around the anus through the neodentate line. Then the proximal bowel is extruded out the anus and (along with the sutures with needles) wrapped in gauze and stabilized. Then after 5-7 d the extruded bowel is amputated and using the already placed sutures, the coloanal anastomosis is completed (Figure 4). This delayed maturation allows the portion of the bowel in the anal canal to adhere to the denuded surface and seal prior to amputation. El-Gazzaz *et al*[6] reported on this technique in 7 patients with CD associated RVF, with a 57.1% healing rate.

Miscellaneous repairs

Tissue interposition: Tissue interposition achieves bringing healthy, well vascularized tissue between the rectal and vaginal walls and acts as a buttress to suture lines as was mentioned in the transverse perineal approach above. Successful use of the gracilis muscle interposition has been reported for Crohn's RVF repairs, especially after other failed repairs. Zmora *et al*[38] reported on their use of the gracilis flap in nine patients for various causes of fistula, including 3 rectourethral fistulae, 1 pouch-vaginal fistula, and 5 RVF (2 CD associated RVF). All patients underwent fecal diversion. Seven patients achieved successful closure with this technique, with 1 CD associated fistulae achieving closure. The authors emphasized the importance of fecal diversion, performing a tension-free rectal repair, and the use of a well-vascularized muscle pedicle. They recommend the gracilis interposition in failed RVF repairs and noted that even though the rate of success in CD is not as high as a surgeon would prefer, a gracilis transposition can be attempted and should be considered. Similarly, in a study by Lefèvre *et al*[39], 4/5 women with Crohn's RVF were successfully closed at a 28 month follow-up, with the use of a gracilis muscle interposition.

The Martius (bulbocavernosus) flap may be used as an adjunct to transperineal repairs with anal sphincter reconstruction (Figure 5). The Martius flap has been reported to improve closure rates and possibly lead to better functional outcomes as well[40]. McNevin *et al*[40] reported on 16 patients with complex anovaginal fistulae, including 2 with CD. They reported success in 15 women and concluded that the Martius flap can be combined with an anterior sphincter repair for complex RVF with minimal morbidity.

Overall there are few studies utilizing the gracilis or Martius flaps in CD RVF. These studies have limited numbers of patients. Therefore it is not clear if the use of gracilis or Martius flaps improves outcomes after RVF repair.

Bioprosthetics: A bioprosthetic fistula plug made from lyophilized porcine intestinal submucosa is a technically feasible option in closing RVF, but the data on its use in Crohn's-related RVF is limited. Schwandner *et al*[41] reported using Surgisis™ mesh in 21 patients with RVF, 9 with Crohn's RVF. After a mean follow up of 12 mo, they achieved a 78% closure rate in the Crohn's group

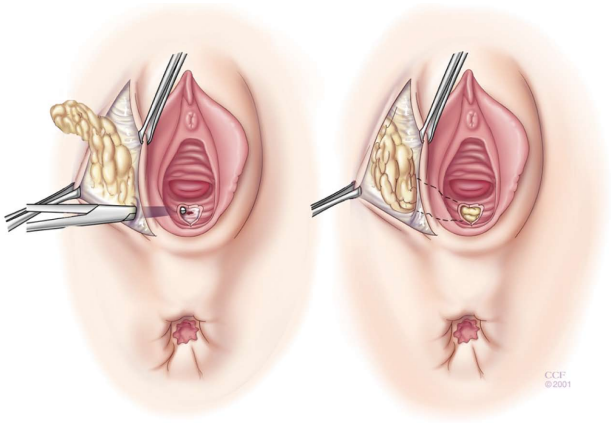

Figure 5 Martius graft. The martius graft begins standard perineal dissection followed by longitudinal incision over the labia majora. Skin flaps are raised medially and laterally until entire fat pad with bulbocavernosus muscle is mobilized. A subcutaneous, subvaginal tunnel is made and the flap is pulled through the tunnel after the anterior end is divided and then sutured to the posterior vaginal wall. Reprinted with permission, Cleveland Clinic Center for Medical Art and Photography © 1999-2014. All Rights Reserved.

and an 83% closure rate in the non-Crohn's RVF. The authors concluded that the mesh plug could be used as an adjunct to traditional advancement flap repair or muscle interposition, or possibly it could be used as an initial operation[41]. In a study by O'Connor *et al*[42], the use of the fistula plug was studied in patients with Crohn's fistulae (2 with RVF) with an 80% success rate. The success of the two RVF was not specifically addressed. Alternatively, in a report from our institution, a retrospective review of 49 plug insertions in thirty-three patients was conducted (13 CD with 2 RVF; 19 cryptogenic origin). The authors reported an 84.6% failure rate for CD associated fistulae (including both RVF) and a 68.4% failure rate for fistulae from cryptogenic origin. These results were much lower than previous reports and the authors concluded that septic complications were the most common cause of failure[43].

Currently, there is little data to support the routine use of bioprosthetics in Crohn's RVF, but the procedure carries a low morbidity and does not preclude further treatments. Further studies are required to determine the role of bioprosthetics in repair of CD associated RVF.

Stem cell transplantation: Adult stem cells extracted from certain tissues can differentiate into different tissue lines, including muscle. In a recent study by García-Olmo *et al*[44] from Spain, a female with Crohn's associated RVF received autologous adipose stem cells that were injected into her RVF. At three month follow up, the patient achieved successful closure of the fistula[44]. This is an exciting potential therapy where further research is needed.

Fecal diversion

As previously mentioned, the use of fecal diversion in repair of Crohn's associated RVF remains controversial. Proximal diversion does control symptomatology and likely improves the condition of the anorectum before subsequent repairs are undertaken. However, equivocal results are obtained whether or not proximal diversion is used in conjunction with RVF repair, regardless of the technique utilized[3]. There are no set criteria regarding when and in whom proximal diversion should be performed. Furthermore, a stoma does not ensure a successful repair. The literature is mixed with recommendations as some authors recommend all patients receive a loop ileostomy before or during repair and others recommend fecal diversion only in select situations. Without any randomized, prospective data, the creation of a stoma remains controversial and surgeons must use their best judgment in making the decision regarding diversion. Our institution recommends the construction of a proximal stoma in the following circumstances: re-do repairs, technically difficult repairs, and suboptimal tissue conditions[3].

Proctectomy

Traditionally, proctectomy has been the definitive treatment of Crohn's related RVF. In an early paper by Tuxen and Castro, total proctocolectomy (TPC) with an end ileostomy was the procedure of choice, due to shortcomings in medical treatment and proximal diversion to heal Crohn's RVF[11]. Over the last several decades, successful repairs with sphincter and rectum sparing techniques have been widely published. Despite published studies of successful repairs for Crohn's RVF, there are still subsets of patients who will require TPC. Patients with extensive colonic involvement or extensive anorectal involvement may not be candidates for definitive repair and proctectomy would be recommended as their initial step in treatment. It should be noted that proctectomy is not without its own complications, as delayed perineal wound healing and the potential for chronic perineal sinuses can be seen in up to 50% of patients in some series[5,22].

Surgical outcomes

Long-term success after repair is not guaranteed regardless of the method used. Crohn's related RVF have a high propensity to recur with a published range between 25%-50%[3,6,45-47]. Most studies have only reported short-term outcomes. Makowiec *et al*[25] evaluated perianal Crohn's fistulae in 32 patients who underwent RAF (12 patients had a RVF). Mean follow-up was 19.5 mo. A recurrence rate in the women with RVF was 58%. The authors analyzed their results which showed a cumulative risk of recurrence at one year of 46% and 72% at 2 years.

Ruffolo *et al*[48] evaluated surgical outcomes in women with Crohn's associated RVF over a 14 year period as well as assessing the effect of anti-TNF-α treatment on healing rates. With various techniques utilized, the authors found a fistula closure rate of 81% in 52 women. The cumulative closure rates after the first, second, third, and fourth attempt at repair was 56%, 75%, 78%, and 81%, respectively[48,49]. Furthermore, primary healing rates were found to be similar in patients receiving ant-TNF-alpha treatment *vs* those who did not.

In a long-term follow-up study from our institution,

El-Gazzaz *et al*[6], studied potential variables that may influence success or failure of fistula closure in Crohn's RVF. We also reported on quality of life and sexual function. With a median follow up of 44.6 mo, 30/65 (46.2%) had successful closure. Repair techniques were as follows: advancement flap (n = 47), episioproctotomy (n = 8), coloanal/Turnbull-Cutait (n = 7), and fibrin glue/plug (n = 3). The authors found that sexual function and quality of life were similar in healed *vs* unhealed women. Predictors of failure included smoking and steroids. The use of immunomodulator medications within 3 mo of repair showed a higher rate of fistula closure[6].

A retrospective study by Athanasiadis *et al*[33] reviewed rates of closure and functional outcomes in Crohn's RVF repair techniques over a 7 year period. Thirty-seven women with RVF underwent 57 operations with various repair techniques. The authors found that techniques with a low degree of tissue mobilization had higher success rates and less postoperative functional problems.

Repair of recurrent fistulae or re-repair of a failed repair is plausible. A review of all methods of repair over nine years for recurrent RVF secondary to all etiologies was undertaken at our institution. An overall success rate of 79% was accomplished after a median of 2 operations. When looking specifically at Crohn's associated recurrent RVF, 6/12 healed after a combined total of 21 operations. The authors noted that the most significant factor to influence outcome of repeat repairs was the duration of time between repairs. Patients re-operated within 3 mo of the original repair had lower healing rate compared to those treated after 3 mo. The authors highlighted that proper patient selection and optimization of clinical conditions is paramount in order to achieve the best possible outcome[46].

CONCLUSION

Rectovaginal fistulae are the most difficult manifestation of perianal CD to treat. They are a source of frustration for the patient and for the treating clinician. A thorough investigational work-up of the entire gastrointestinal tract, the anal sphincters, and the anorectum must be performed before any treatment attempt can be undertaken. Only after failed medical management and when local conditions are suitable, can surgical intervention be contemplated. Initial control of perianal sepsis with drainage and possible seton placement is paramount and may be the only treatment required. Medical treatments are indicated to control both local and distant active CD. Immunomodulators and anti-TNF-α therapy may play a role in primary correction of fistulae or may be used as an adjunct to surgical repairs. The surgical management of RVF can be complex and the treatment plan must be individualized. The chosen technique is based on the anatomy of the fistula, patient symptoms, and quality of life. The experience of the surgeon also influences the choice of repair and multiple options must be in one's armamentarium. Often, repairs fail and reoperative intervention is necessary, with acceptable results. Maintaining realistic treatment goals and expectations is essential.

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REVIEW

Zinc and gastrointestinal disease

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Abstract

This review is a current summary of the role that both zinc deficiency and zinc supplementation can play in the etiology and therapy of a wide range of gastrointestinal diseases. The recent literature describing zinc action on gastrointestinal epithelial tight junctions and epithelial barrier function is described. Zinc enhancement of gastrointestinal epithelial barrier function may figure prominently in its potential therapeutic action in several gastrointestinal diseases.

Key words: Zinc; Tight junction; Nutrition; Nutraceutical; Micronutrient

Core tip: This is an overview of the role that both zinc deficiency and zinc supplementation can play in the etiology and therapy of a wide range of gastrointestinal diseases.

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INTRODUCTION

When considering the topic of zinc and gastrointestinal (GI) disease, several general biomedical and nutritional situations must be considered. The first, and perhaps most obvious, is that of dietary zinc deficiency (ZD) - whether this arises out of generalized diet insufficiency, genetically-based zinc malabsorption, or dietary interference with zinc absorption (*e.g.*, phytates in the diet) - and resultant diseases from said deficiency. The second consideration is that diseases, such as the inflammatory bowel diseases (IBDs), whose morbidity *generates* GI malabsorption issues, ultimately giving rise to ZD. Finally, one must consider the *supplementation* of zinc to the diet, and the positive role that may play in certain GI diseases, especially those characterized by impairment of barrier function. The connection among all three situations is perhaps that ZD, from whatever source appears to lead to GI barrier compromise, an eventuality that is self perpetuating (Figure 1).

This is, then, a very broad topic, and one in which numerous excellent reviews have been written concerning the above individual situations. Duggan *et al*[1] (2002) did a thorough reporting of zinc and other "functional foods" for maintaining GI mucosal function. In terms of barrier function per se, Hering *et al*[2] (2009) have recently published on this from a more cellular perspective. Semrad[3] (1999) reported on the general role of zinc in intestinal function, particularly in diarrhea. Goh *et al*[4] (2003) deal with both ZD arising out of IBDs as well as the role zinc and other nutraceuticals may play in providing an alterna-

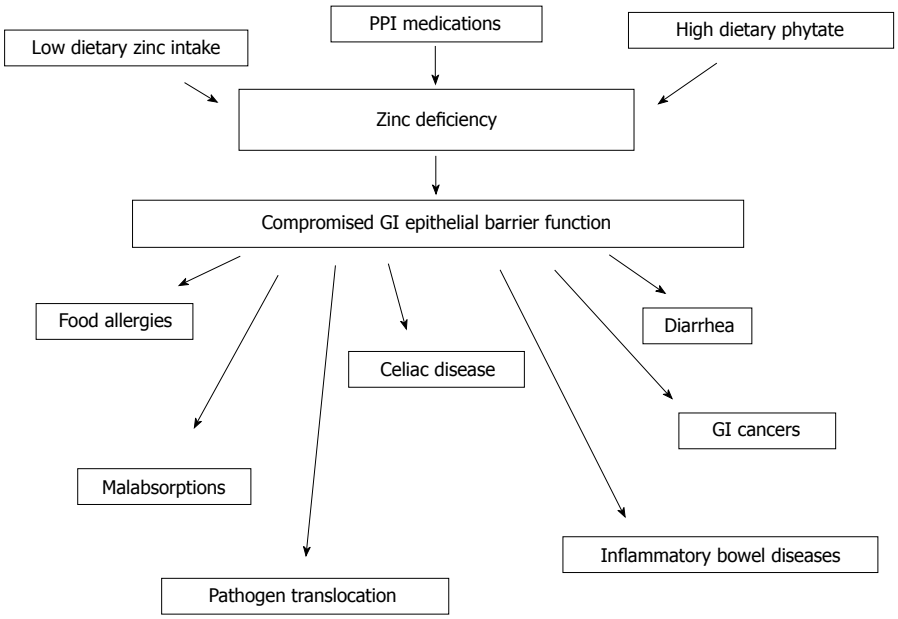

Figure 1 Zinc deficiency can arise from several sources, and a major physiological effect of zinc deficiency will be to induce leakiness in tight junctional seals and consequently epithelial cell layers. This figure diagrammatically shows the conditions/diseases that could be promoted by this eventuality arising in the gastrointestinal mucosa; GI: Gastrointestinal; PPI: Proton pump inhibitor.

tive to the use of steroids and anti-tumor necrosis factor (TNF) modalities in IBD therapy. Treatment *via* zinc supplementation of GI disease incited by ZD may in fact be the first (though inadvertent) clinical summary of supplemental zinc effects on GI barrier compromise[5]. The very concept of ZD as well as the myriad roles played by zinc in cellular and systemic function, are discussed comprehensively by Tuerk *et al*[6] (2009) and Wapnir[7] (2000). The singular issue of zinc in parenteral feeding, an important medical area for which zinc (and epithelial barrier function) may be highly important, is something that we do not consider here in any depth, but has been well investigated by Jeejeebhoy[8] (2009). The critical area of zinc "physiology", *i.e.*, its transport and binding in cells in general, and enterocytes in particular [ZIPs, ZnTs, metallothioneins (MTs), *etc.*], as well as the issue of zinc homeostasis systemically is discussed thoroughly by King[9] (2010), King *et al*[10] (2000) and Cousins[11] (2010). Finally, a very comprehensive review of zinc's role in disease generally has just recently been produced[12].

Our review generally does not address the enormous literature surrounding zinc finger proteins and zinc metalloproteinases, except where these proteins specifically deal with a deficiency or supplementation. There are numerous reviews covering these various topics touching on zinc[13-16]. Both protein classes have almost certain relevance toward all of the diseases we discuss in this review, but we largely avoid that literature because of its focus on the protein rather than specifically on zinc.

As alluded to above, one can be ZD for three reasons: (1) the diet is simply too low in zinc content, as can be true for certain diets poor in meat or fish protein; or (2) and (3) there can be two situations of ZD in the face of diets replete with zinc. The latter two reasons can be brought about through the chronic use of Proton Pump Inhibitor (PPI) medications, whose inhibition of gastric acid production (and elevation of gastroduodenal lumen pH) can render luminal zinc non-absorbable[17]. In addition, diets high in the form of phosphorous known as phytate (found significantly in whole grains, nuts and seeds), can result in zinc complexing with phytates and a resultant non-absorbable species[18,19]. It is worth considering that the average American, especially those of higher socioeconomic status, may be the victim of one or both outcomes, as PPIs are taken by over 20 million Americans yearly, and phytates exist at high levels in the very foods that are prominently featured in "healthy diet choices". The now common use of PPIs to treat gastroesophageal reflux in neonates makes this group particularly worrisome in this regard. Additionally, the body has no cell/tissue zinc stores (unlike, *e.g.*, iron or calcium) therefore its daily zinc needs are heavily dependent on satisfactory daily zinc intake and at least short-term states of zinc deficiency could be quite likely[20].

The government recommended daily intake of zinc is 11 mg/d[21], however studies have shown that, at least in the relatively short term, higher doses of zinc are safe. For example, our recent study in patients with Barrett's esophagus showed no adverse effects on a total dose of 52 mg/d for 14 d, with positive changes on intracellular signal transduction in the Barrett's epithelia[22]. It is clear that a dose of 150 mg/d over an extended period can be toxic, as is evidenced by the development of severe copper deficiency and anemia[23,24], but zinc lozenges are only effective in reducing the duration of the common cold at a minimum dose of 75 mg/d[25]. At daily doses above 50 mg/d, periodic measurements of blood copper levels (which can be driven lower by high zinc intake) and cho-

Table 1 Gastrointestinal morbidities associated with Zinc deficiency

Condition	Model used	Reversibility with Zn supplementation	Ref.
Esophageal cancer	Rodent	Yes	Fong *et al*[27], 2011
Diarrhea	Human	Yes	Hambidge[72], 1992
Inflammatory bowel diseases	Porcine	Yes	Sturniolo *et al*[82], 2002
Celiac disease	Human	Not determined	Wierdsma *et al*[143], 2013
Alcoholic liver disease	Mouse	Yes	Lambert *et al*[198], 2003
Malnutrition	Guinea pig	Yes	Rodriguez *et al*[65], 1996

lesterol levels would be prudent[26].

The use of zinc as a potential therapeutic in these various diseases and disorders should be treated realistically. No one would suggest, *e.g.*, that zinc-induced potentiation of GI barrier function might "cure" any associated illnesses. However, considering the issue quantitatively rather than qualitatively, there is a quite reasonable possibility that zinc administration could reduce morbidity by a meaningful extent, and that would be highly useful. This is especially true given that zinc could readily be used together with existing medications for these conditions.

The role that zinc may exert in specific GI diseases is now discussed below, along with a final consideration of zinc's role in maintaining GI epithelial barrier function. The compromise of that barrier function can be a catalyst for several of these diseases, and zinc-induced barrier enhancement may prove to be a means of reducing morbidity in some of these conditions (Table 1).

An important consideration in reading this review and interpreting the findings that are presented, is that one needs to always consider the chemical form of the administered zinc, its dosage/concentration, and its vehicle (tablet, capsule, emulsion, lozenge, *etc.*), in the interpretation of any given result/finding. Not all zinc salts are equally soluble and therefore permissive for zinc delivery to cells. In addition, in the discussion presented below on zinc-mediated inhibition of esophageal cancer in rodents, it is critical that the zinc was administered in drinking water[27]. If it was administered in capsule or tablet form, it would result in no topical delivery to the target tissue. This is further discussed in Valenzano *et al*[22] (2014) for delivery to humans. It is not really feasible in a review to discuss the mode of zinc administration in every citation, and so we encourage the reader to refer to specific references for chemistry, drug delivery and concentration information.

ZINC AND GASTROINTESTINAL CANCER

While there is a minimal amount of published literature regarding zinc's effect on transformed cells in culture, zinc - at a concentration that is otherwise not seen to be toxic or a hindrance to normal cell growth - has been shown to negatively impact growth in transformed human tumor cells[28]. Further evidence of tumor growth inhibition by zinc was observed in mice inoculated with sarcoma 180 cells into the peritoneal cavity. Mice treated with zinc sulfate injections experienced a suppression in the number of tumors produced; however, the treatment was unable to prevent the subsequent growth of a tumor once it developed[29]. Similarly, when mice inoculated by intraperitoneal injection with L1210 leukemia cells were treated with zinc acetate injections, they exhibited a reduction in tumor cell growth[30]. The mechanism by which zinc is capable of producing this effect may relate to its ability to prevent oxidative stress that causes DNA damage. Additionally, it is noteworthy that ZD impairs DNA repair by compromising the DNA binding activity of the tumor suppressor protein, p53, disrupting its function[31,32]. In a study on the treatment of the human colon cancer cell line, HCT-116, with zinc chloride, zinc inhibited cell proliferation by stabilizing adenomatous polyposis coli (APC) protein and arresting cell growth[33]. Zinc dysregulation and its subsequent effect on the development of cancer has been suggested to be cell type specific. For example, breast tumor biopsies have higher zinc levels than normal tissue whereas cellular zinc levels in prostate cancer and ovarian cancer tumor tissue are significantly lower than benign tissue[34].

Esophageal and oral cancers are significant upper aerodigestive tract cancers. Esophageal cancer is the eighth most common cancer and the sixth most common cause of death from cancer worldwide, while oral cancers, particularly those of the tongue, have a high mortality rate in part due to the risk of developing a second primary tumor, usually in the esophagus[35,36]. Esophageal cancers can be either squamous cell carcinoma (SCC) or adenocarcinoma (AC). Abnet *et al*[37] (2005) performed a 16-year observational study on participants in a nutrition intervention trial and found that the subjects who had a high zinc concentration in their esophageal biopsy specimens also had a reduced risk of developing esophageal SCC, indicating that dietary ZD associates strongly with SCC. Although studies examining dietary zinc intake and its relationship to cancer risk have reported conflicting results, a meta-analysis of 19 studies involving an estimated 400000 participants found that the level of zinc intake was inversely associated with digestive tract cancers, especially colorectal cancer[38]. In addition to these cancers of the GI tract, low serum zinc levels are also associated with prostate, ovarian, lung, gallbladder, and head and neck cancers[34].

There is strong evidence that ZD in rats and mice enhances carcinogenesis. When control and ZD rats were intragastrically administered 2 mg/kg body weight doses of the carcinogen methylbenzylnitrosamine the ZD rats

had a higher frequency of esophageal tumor development[39]. Similar outcomes ensued when ZD and zinc-sufficient (ZS) Sprague-Dawley rats were exposed to N-nitrosodimethylamine. The control rats did not suffer epithelial irregularities; however, 63% of the rats deficient in zinc developed squamous papillomas in the forestomach[40]. ZD and ZS Sprague-Dawley rats, exposed through their drinking water to precursors of the carcinogen N-nitro-N-benzylmethylamine (benzylmethylamine and $NaNO_2$) manifested like results[41]. Similarly, when C57BL/6 mice were given N-nitrosomethylbenzylamine (NMBA), after 46 wk the ZD mice had significantly more esophageal and forestomach tumors than ZS mice[42]. In an oral cancer study, ZD and ZS rats were treated with the carcinogen 4-nitroquinoline 1-oxide (NQO) to induce lingual tumors. A greater incidence of lingual squamous cell carcinomas was found in the ZD rats[43]. This is not a phenomenon limited to squamous cell cancers, as zinc supplementation (in combination with aspirin or vitamin C) showed dramatic reduction of colon tumors in dimethylhydrazine-treated rodents[44].

The mechanisms by which ZD creates a pro-tumor environment may relate to its ability to induce cell proliferation. In a study on the relationship between cell proliferation and tumor incidence, ZD and ZS rats either received intragastric doses of NMBA or were left untreated. At various time points, *in vivo* bromodeoxyuridine (BrDU) labeling and immunohistochemical detection of cells in S-phase were used to assess esophageal cell proliferation. In both NMBA-treated and untreated rats, the ZD condition showed a significantly higher labeling index than the ZS condition. In NMBA-treated animals, 100% of the ZD ad libitum rats, 23% of the ZS ad libitum fed rats, and 6% of the ZS rats pair-fed to the ZD rats developed tumors. After about 10 wk of the ZD diet, two rats not exposed to NMBA developed esophageal papillomas[45]. In an alternate study, BrDU labeling of ZD and ZS mice given doses of NMBA intragastrically showed that the labeling index and number of labeled cells were also increased in the ZD mice[42].

Dietary ZD also alters gene expression. Liu *et al*[46] (2005) identified 33 genes that were differentially expressed in a hyperplastic ZD *vs* a ZS esophagus. Key factors are the upregulation of the cyclooxygenase (COX-2) inflammatory gene and the induction of an overexpression of the proinflammatory mediators, S100A8 and S100A9. In the hyperplastic esophagus and tongue of ZD rats, the expression levels of both COX-2 protein and mRNA were between 8 and 14.6 fold higher than their ZS counterparts[43]. Treating these rats with an inhibitor of the COX-2 pathway, celecoxib, led to a reduction in cell proliferation but not a prevention of carcinogenesis, suggesting that there must be an additional process involved[43,47]. Celecoxib was found not to be an efficient treatment because it did not show a real effect on S100A8 overexpression. The expression of S100A8 and S100A9 in hyperplastic ZD esophagi was upregulated 57 and 5 fold, respectively[48]. Combining ZD-induced inflammation with low levels of NMBA resulted in a 66.7% incidence of esophageal SCC[49].

ZD in collaboration with other factors, such as p53 deficiency and cyclin D1 overexpression, can produce an accelerated progression towards malignancy[50-52]. p53 is a tumor suppressor protein responsible for the prevention of uncontrolled cell proliferation. Both p53 deficiency ($p53^{-/-}$) and insufficiency ($p53^{+/-}$) in combination with ZD leaves mice more susceptible to carcinogens, increasing the tumor incidence in the esophagus and tongue[50,52]. This rapid rate of tumor progression was accompanied by nearly 20% of ZD and p53-deficient rats developing esophageal Barrett's metaplasia[50]. Cyclin D1 overexpression in conjunction with ZD disrupts the cell cycle leading to uncontrolled cell proliferation and consequently a substantial increase in tumor incidence. At 77 d, 14% of mice with both cyclin D1 overexpression and ZD developed esophageal intestinal metaplasia[52].

Several experiments have investigated the ability of zinc replenishment (ZR) to prevent esophageal cancer. ZR was shown to begin reversing the inflammatory signature and reduce COX-2 overexpression to only 3-fold of that seen in ZS rats[43,49]. ZR also stimulated apoptosis, increased the expression of the proapoptotic Bax protein[53], returned 29 of the 33 altered genes back to normal expression levels[54], decreased cell proliferation[55], restored the expression of S100A8 and S100A9[48], and consequently began to reverse the pre-cancerous phenotype condition. Furthermore, animals with an already ZS diet were found to benefit from zinc supplementation. Additional zinc in the diet of these rats limited cell proliferation and stimulated apoptosis causing a reduced incidence of tumors and tumor progression induced by both low and high doses of NQO[27]. This finding is critical support for the value of zinc supplementation in cancer chemoprevention.

Dietary ZD may be creating a pro-tumor environment in the GI tract, enhancing carcinogenesis by inducing cell proliferation, altering gene expression, and promoting inflammation. Zinc as a dietary nutrient is essential because it plays a role in a variety of important functions, including DNA repair, apoptosis, cell cycle progression, p53 activation, and the prevention of oxidative stress that causes DNA damage. This in conjunction with several research studies suggesting zinc replenishment and supplementation results in a positive effect against carcinogens, supports the thesis that zinc supplementation has the potential to be efficacious in the prevention of several GI cancers.

ZINC AND DIARRHEA

It is well described that ZD can lead to diarrhea. Acrodermatitis enteropathica, a hereditary disorder of zinc metabolism, was reported in infants with skin lesions and diarrhea in the early 1970s[56], and shortly thereafter a similar syndrome was found in certain adult patients placed on total parenteral nutrition (TPN). These patients presented with diarrhea, depression and dermatitis, and

were found to have acute ZD[57]. Zinc supplementation resulted in rapid improvement of diarrhea and dermatitis in both of the above groups[56-61]. Very recent meta analyses of multiple studies supports this conclusion, showing decreased diarrhea duration with zinc therapy[62,63]. A recent review on infectious diarrhea supports this[64]. It is noteworthy that even in control, non disease states, zinc supplementation can positively affect multiple aspects of GI mucosa (on a molecular and cellular level) that would likely act to enhance GI barrier function[46]. ZD can cause intestinal hyperpermeability ("leaky gut"), which itself can be secondary to increased nitric oxide and oxidative stress, thereby leading to diarrhea[65-67]. In rats, ZD has been shown to upregulate expression of intestinal uroguanylin (a peptide that triggers Cl^- secretion and subsequent water secretion)[67,68], decrease absorption of triglycerides by altering chylomicron formation[69], and decrease absorption of proteins by altering enterocyte peptidase activity[70,71], all of which can potentiate diarrhea.

Later it was discovered that not only can ZD cause diarrhea but also chronic diarrhea conditions can cause ZD, thereby promoting even more diarrhea[72]. This has led to multiple studies of zinc supplementation in infants and children with diarrheal illnesses, mostly in developing nations where malnutrition often results in ZD. Children with acute diarrhea treated with zinc show a decrease in the rate and duration of diarrhea as well as decreased need for antibiotics when compared to controls (who were typically treated with oral rehydration and/or vitamin supplements)[73-76]. Zinc supplementation in healthy children in developing countries has decreased the prevalence, morbidity and mortality of diarrhea[77]. The World Health Organization currently recommends treating diarrhea in children with zinc tablets along with oral rehydration solutions as part of a first-line approach[78].

ZD can also decrease human immune function, increasing the risk for infection[74,79]. In infectious diarrhea there is typically increased intestinal permeability (seen as an increase in the lactulose/mannitol ratio)[80] which can sometimes be improved by zinc supplementation[81-83]. ZD leads to decreased electrolyte and water absorption, and can exacerbate diarrhea caused by *Vibrio cholera* (*V. cholera*)[84,85]. *V. cholera* causes diarrhea by increasing cyclic adenosine monophosphate (cAMP) production, inducing the intestine to secrete water and chloride, and inhibiting the absorption of sodium[85]. Interestingly, zinc supplementation actually impedes cAMP-regulated secretion of chloride *via* basal-lateral K^+ channels, explaining its efficacy in reducing the duration of cholera-induced diarrhea, an effect that may involve basal-lateral zinc action on basal-lateral membrane K^+ channels[86,87]. Zinc supplementation decreases expression of certain genes linked to immune function in piglets infected with enterotoxigenic *E. coli* (ETEC) as well decreasing ETEC-induced diarrhea and inflammation. This action is possibly due in part to a decrease in MUC4 expression, which might be an ETEC K88 receptor[88]. In treating ETEC-related diarrhea the mode of delivery in zinc supplementation can matter significantly[89]. In CACO-2 cells, zinc supplementation inhibits Ca^{++} and nitric oxide-mediated ion secretion, both of which are known pathways for pathogen-induced diarrhea. However the same may not be true for Ca^{++}-mediated ion secretion in rat ileum[86,90]. It should be noted however that Bzik *et al*[87] (2012) did observe an inhibitory effect of zinc on carbachol-stimulated short circuit current. Zinc has also been shown to have a direct antimicrobial effect on infectious enteric bacteria such as *E. coli*, *Shigella*, and various strains of *Salmonella in vitro*[91].

In summary, zinc is useful in the treatment of diarrhea of various etiologies. Its role in decreasing fluid secretion to the intestinal lumen (directly or indirectly) requires further investigation but cannot be disputed. Its impact on intestinal paracellular leak, another potential source of diarrhea, will be addressed in a later section of this review.

ZINC AND CROHN'S DISEASE

ZD has been well established in Crohn's disease (CD) and can arise in part due to poor zinc absorption in the small intestine (even if the jejunum appears normal) as well as from chronic dietary intolerances and restrictions[92-96]. In a large cohort of patients with IBD, it was estimated that 8.5% of patients had inadequate intake of zinc. The prevalence of low serum zinc levels was 29.3%[97]. ZD has even been documented in Crohn's patients while in remission[98]. Crohn's patients on TPN can develop acute ZD resulting in acrodermatitis enteropathica and decreased vision[99]. More commonly, ZD in CD contributes to stunted growth in children and manifests as decreased taste sensation, visual acuity, and immune function[100-102].

One very important element of CD pathophysiology is a defect in mucosal barrier function. Increased mucosal permeability correlates with disease activity as evidenced by increased uptake of large molecular markers (such as lactulose) from the GI lumen into the bloodstream[103]. While in remission, increased transmucosal permeability (leak) has been used as a marker for predicting relapse[104]. In the human intestinal cell line, CACO-2/T7, zinc depletion in conjunction with TNFα exposure (common in the inflamed mucosa in CD) increases apoptosis, ultimately compromising the organization of tight junctions (TJs) and epithelial barrier integrity[105]. This could explain why zinc supplementation has been reported to decrease transmucosal leak in CD[106], although this finding has been challenged[107]. CD pathophysiology involves a persistent recruitment of leukocytes into intestinal mucosa and submucosa followed by an unregulated granulomatous inflammatory response. Epithelial barrier dysfunction may facilitate this passage of leukocytes and allow for an unregulated leakage of luminal antigens across the epithelial barrier, a leak that normally does not take place. This is evidenced by a high frequency of neutrophils and crypt abscesses in the intestinal mucosa[108]. ZD may exacerbate CD morbidity by increasing gut permeability,

allowing for increased neutrophil transmigration and luminal antigen permeation[109]. Biologic agents such as Natalizumab have taken advantage of this persistent recruitment mechanism by blocking leukocyte transmigration at the level of the vascular endothelium[110,111].

Zinc supplementation in CD, whether the Crohn's is active or in remission, may be beneficial. It had been postulated that zinc supplementation in patients with IBD would increase serum and mucosal levels of the zinc-dependent free radical scavengers superoxide dismutase (SOD) and MT, however their rise in concentrations was not statistically significant[112]. According to Brignola *et al*[113] (1993), dietary supplementation with zinc in CD patients with active disease significantly improved serum zinc levels in addition to levels of zinc-dependent hormones like thymulin, which can potentiate T-cell differentiation and natural killer (NK) cell activity[113]. The effect of zinc supplementation on NK cell activity of IBD patients, varies in the literature. A small randomized trial by Van de Wal *et al*[114] (1993) showed a significantly reduced level of NK cell activity with zinc supplementation. Despite the current evidence in the literature for their benefits, zinc products have not undergone the scrutiny and validation of the Food and Drug Administration for this disease. For this reason, there are no specific guidelines for zinc supplementation beyond emphasizing the importance of adequate and balanced nutritional intake in CD. As described in the sections on zinc and GI cancers, and zinc and TJs, there may well be medical benefit of zinc daily intake above the current RDA.

ZINC AND ULCERATIVE COLITIS

Zinc is absorbed from the GI lumen principally in the small intestine at the distal duodenum and proximal jejunum[115]. This leads to obvious implications for zinc malabsorption in diseases targeting and damaging the small intestine, such as CD and Celiac sprue. However, ulcerative colitis (UC) does not typically manifest in the small bowel proximal to the ileum, and numerous studies have shown that well nourished UC patients are not ZD. In fact, when patients with moderate and severe (active) UC are compared to healthy controls and/or UC patients in remission, they have shown normal or even elevated serum zinc concentrations[112,116,117]. Similarly, serum zinc levels in children with UC were no different than controls[118].

In active UC flares, elevated serum zinc concentrations are correlated with increased levels of complement component C3C (part of the innate immune system) and elevated antinuclear antibodies, a known predictor of UC flares and a marker of steroid dependence in UC[119,120]. This may be indicative of zinc being released locally in response to the activated inflammatory cascade in active UC. However, an additional study showed no correlation between serum zinc and elevated erythrocyte sedimentation rate or C-Reactive protein, both standard predictors of high grade inflammation in UC[121]. Patients with refractory UC who underwent colectomies with ileal pouch anal anastomosis or a Brooke ileostomy had either elevated or normal serum zinc levels[122-124]. In fact, ZD in UC patients most likely stems from malnutrition deriving from poor oral intake due to acute (systemic) illness associated with active UC and not the damaged colonic tissue *per se*[125,126]. There is little evidence that the colon itself normally has any major role in zinc metabolism or homeostasis.

On a molecular level, the reactive oxygen species (ROS) produced from respiratory burst during neutrophilic invasion in UC cause significant mucosal damage[127]. SOD, vitamin E and ascorbic acid are all known to reduce ROS species produced in this process[128]. Zinc in various ionic forms and as a part of various moieties in proteins is also important in ROS reduction. For example, the copper/zinc isoform of SOD (Cu/Zn-SOD) removes ROS and indirectly prevents lipid peroxidation[129]. Interestingly, Cu/Zn-SOD is decreased in the epithelium of active UC[130]. Another family of zinc-cytoprotective enzymes are MTs. These are zinc-binding proteins that likely play a role in alleviating acute inflammation as ROS scavengers. The literature shows variability in MT expression in IBD. In ZD individuals, mucosal layers of active UC patients had decreased concentrations of MTs in their colonic mucosa and had increased concentrations of ROS intermediates[131-133]. Inducing ZD in animal models of UC increases the disease activity index (DAI) and serum TNFα levels, exacerbates weight loss, and leads to further shortening of colon length[134]. MT concentrations are increased in UC-associated colorectal carcinoma but are decreased in active UC flares at the inflamed mucosa[116,135]. In actively inflamed UC in both human and animal models, supplementing zinc has been shown to either slightly increase or have no effect on the tissue level of MTs[112,136-138]. There have been many studies on the effects of zinc supplementation on inflammation in UC. Di Leo *et al*[136] (2001) reported improvements in diarrhea and weight gain in a rat colitis model when supplemented with zinc but found no effect on neutrophil infiltration or visible inflammation. Mulder *et al*[112] (1994) reported no change in DAI or inflammation in human UC intestinal biopsies after zinc supplementation. More recently, however, Tran *et al*[137] (2007) found that zinc supplementation suppressed colitis in a mouse model as evidenced by decreased DAI and histological severity scores as well as reduced myeloperoxidase activity. Intrarectal zinc also has been found to be beneficial at the microscopic level with reduced inflammation in rat models in as little as 96 h, with other studies also reporting similar decreases in inflammation in both rat and mouse UC models[139-142].

In summary, in UC patients, systemic ZD does not arise in response to colonic tissue inflammation, but if a patient has decreased nutritional intake due to associated illness in ongoing UC, they may develop insufficient levels of serum zinc. This decreased serum zinc concentration does appear to have important implications at the cellular level and therefore may diminish the body's ability

to reduce active inflammation. More research is required to further examine the benefits of zinc supplementation in UC patients, specifically its effect, if any, on epithelial barrier function.

ZINC AND CELIAC DISEASE

Celiac disease (CeD) is an immunologic enteropathy triggered by the intake of gluten (and more specifically gliadin) which causes, among other outcomes, the loss of brush border proteins, enzymes needed for both digestion and absorption. CeD patients are deficient in many vitamins and minerals, including zinc[143]. Decreased plasma zinc has been observed in both untreated CeD patients and as well as those in clinical remission[144]. The prevalence of ZD in newly diagnosed adult CeD patients has been reported to be as high as 67%, and up to 64% of children with CeD are also ZD[143,145-148]. Moreover, ZD is known to correlate with villous atrophy, with one study finding 60% of patients with partial villous atrophy, 80% of patients with subtotal villous atrophy, and 92% of patients with total villous atrophy to be deficient in zinc[149-152].

Adherence to a gluten free diet (GFD) typically induces clinical and histological remission of CeD. While serum zinc levels are significantly lower in children with untreated CeD with enteropathy, levels normalize after following a GFD[147,153,154]. Interestingly, Jones *et al*[155] (2010) found that supplementing zinc along with a GFD provided no additional rise in plasma zinc levels. A small study in patients with non-responsive CeD showed a rise in serum and plasma zinc after oral zinc supplementation as well as a slight increase in brush border enzyme activity, but there was no improvement in the patients' clinical status[155]. Both Crofton *et al*[156] (1990) and Tran *et al*[157] (2011) found no difference in zinc absorption between untreated CeD patients and controls, however there was evidence of impaired zinc homeostasis. Crofton *et al*[156] (1990) reported an "increased turnover and loss of endogenous zinc" that improved on a GFD, and Tran *et al*[157] (2011) found the exchangeable zinc pool (EZP), *i.e.*, the total of the zinc pools in the body that are able to exchange with serum zinc, to be significantly decreased in CeD patients. Since the size of the EZP directly correlates with zinc nutritional status, patients with CeD can be ZD despite normal zinc absorption[157-159].

Stenberg *et al*[160] (2008) suggest that ZD, specifically in the intestinal mucosa (perhaps due to recruitment of zinc to a site of inflammation elsewhere in the body), activates the enzyme transglutaminase-2 (TG2) (normally *inhibited* by the presence of zinc) in the intestine. They theorize that a TG2-thioester intermediate-deamidated gliadin complex acts as a "neo-antigen," activating T-cells in persons genetically susceptible to CeD. These activated T-cells trigger B-lymphocytes to make antibodies against both TG2 and gliadin with the resultant inflammation causing villous atrophy (2008). Possibly contributing to this inflammatory phenomenon is an increased baseline level of paracellular permeability to gliadin *via* the TJs of the gut[161,162]. As the inflammation response intensifies, enterocyte apoptosis occurs and the TJs become increasingly disorganized, further amplifying paracellular permeability[163-165].

It remains to be investigated whether the known ability of zinc to enhance intestinal epithelial TJs[166] (see below) could be a therapeutic modality in CeD by way of reducing gluten and other luminal antigen permeation across the small bowel epithelium of the CeD patient.

GASTRIC CYTOPROTECTION AND INHIBITION OF ACID SECRETION BY ZINC

The observation of zinc affording protection of the gastric mucosal surface was actually reviewed over 20 years ago[167]. Bandyopadhyay *et al*[168] (1997) reported in animal studies that orally-administered zinc protects against chemically-induced ulceration, presumably in part by inhibiting gastric H^+ secretion. This followed published observations of zinc-mediated suppression of gastric acid output as well as zinc-derived improvement of gastric ulcer healing in the 1970s and 1980s[169-171]. Kirchhoff *et al*[172] (2011) more recently reported in humans that low-dose zinc administration abolishes secretagogue-induced gastric acid secretion, raising luminal pH as effectively as PPI medications. It remains to be determined if orally-administered zinc can be as effective as PPIs in treating, *e.g.*, gastroesophageal reflux disease without at least some of the side effects (liver cytochrome P450 inhibition) of the omeprazole class of drugs. These actions of zinc may trace in part to zinc's observed stabilizing effect on secretory cells and lysosomal organelles in general[173].

GASTRIC ACID SUPPRESSION AND ZINC DEFICIENCY

Omeprazole-induced inhibition of gastric acid production and elevation of gastroduodenal luminal pH has been observed to reduce small intestinal zinc absorption in humans[17,174], although not all studies agree[175]. Joshaghani *et al*[176] (2012) reported lower serum stores of zinc in males after 8 wk of omeprazole use and decreased zinc absorption was also observed with inhibition of gastric acid production by H-2 blockers[177]. The ability of omeprazole to increase pH only extends to the lumen of the stomach and upper small intestine[178]. It should be noted that the duodenum is not the only site for zinc absorption, as the cecum and colon have been observed to contribute significantly to zinc absorption by the GI tract if small intestine absorption is impaired, though the extent of absorption is not as great[179].

ZINC AND EPITHELIAL BARRIER FUNCTION

In most of the GI morbidities discussed in this review, the role of transepithelial barrier leak specifically at the site of the epithelial TJ, is very prominent. The recently documented ability of zinc to reduce TJ leak is very likely involved in zinc supplementation's ability to alleviate these morbidities, and the exacerbation of the morbidities in periods of ZD. For this reason, we present here an expanded description of zinc action on the tight junctional complex.

Examining the impact of ZD or supplementation on epithelial barrier function requires one to consider the molecular structure of the barrier. Epithelial and endothelial TJs (zonula occludens) selectively seal the space between adjacent cells, preventing unregulated paracellular exchange across the epithelial or endothelial barrier. The TJs are comprised of various proteins including the tetraspan transmembrane proteins occludin, tricellulin, and the 24+ members of the claudin family, as well as the single span transmembrane protein JAM-A, scaffolding proteins zona occuldens (ZO)-1, ZO-2, ZO-3, and ZAK and various peripheral membrane proteins[180,181]. Additionally, the TJ is regulated by the actin cytoskeleton through its interactions with scaffolding proteins[182]. The TJ's primary role is to regulate the paracellular permeability of the epithelial or endothelial barrier. Once thought to be a completely passive structure, it is now known that the TJ is actually quite dynamic, constantly adapting to stimuli[183]. Claudins are thought to interact in both homophilic and heterophilic patterns. Certain interactions are likely to have a sealing function while others can form pores that can be anion or cation specific[184,185]. Occludin likely plays a regulatory role at the TJ based on its phosphorylation state[186]. Exposure to different environmental stimuli such as pathogenic bacteria, foods or micronutrients can have a drastic positive or negative effect on the TJ's ability to regulate permeation of nutrients, water, and electrolytes[183].

The presence or absence of zinc has been shown to impact the barrier on its own, in conjunction with other molecules, and in various disease states. Zinc is a divalent cation that plays an important role in many mechanisms in the body. It is involved in DNA replication and transcription to RNA (*via* zinc-finger transcription factors), metalloproteinase catalysis, protection from oxidative stress (when bound to MTs), regulation of apoptosis, cell homeostasis, and immune function[187-191]. Both zinc supplementation and zinc deprivation have been found to impact epithelial barrier function with some evidence indicating this effect is at least partially due to TJ modifications[109,192]. In addition to TJ modification however, epithelial barrier leak can be induced more simply and dramatically by induction of cell death and detachment. Zinc has been shown to be quite able to induce apoptosis at certain concentrations in certain cell types[193,194]. However it has also been shown that epithelial barrier function can withstand the onset of cellular apoptosis (on an individual cell basis) by phagocytosis of the apoptotic cell by its epithelial neighbors, a process that ensues throughout with preservation of tight junctional seals[195].

The following disease situations illustrate zinc's ability to modify TJs in medically relevant scenarios.

Alcohol toxicity

Chronic alcohol exposure has drastic consequences for the liver. Alcohol induces leakage of endotoxins from naturally occurring Gram negative bacteria across the epithelial barrier of the gut into the bloodstream[196]. Alcohol-fed mice have impaired barrier function of the ileal epithelia (as measured by plasma endotoxin levels and increased leak of fluorescein-labeled dextrans) and a correlating decrease in levels of certain TJ proteins such as occludin and ZO-1[197]. Zinc supplementation has been shown to improve and even reverse alcohol induced epithelial barrier permeability and liver damage. Pre-treatment with zinc (prior to alcohol consumption) of a mouse model that mimics binge drinking in humans keeps serum levels of endotoxin at levels close to normal, resulting in significantly less elevation of serum ALT, AST and liver TNF-α, thereby inhibiting liver necrosis[198].

Alcohol exposure is also known to increase ROS in the ileum which is itself associated with decreased ileal zinc concentration. CACO-2 (human GI epithelia) cells exposed to alcohol display a time-dependent increase in barrier permeability (as shown by decreased R_t and increased flux of fluorescein-labeled dextrans) accompanied by an increase in ROS. When zinc deprivation is induced in CACO-2 cells by the zinc chelator, TPEN, there is significant downregulation of claudin-1, occludin and ZO-1. The combination of mild zinc deprivation and alcohol exposure to CACO-2 cells results in even greater decreases in expression of claudin-1, occludin, and ZO-1 as well as impairment of barrier function (as measured by increased dextran leak and decreased R_t)[192]. hepatic nuclear factor α (HNFα) is a zinc finger nuclear transcription factor regulating the expression of certain genes in the kidney, intestine, and liver that is highly expressed in the ileum. The alcohol-induced increase in ROS inactivates HNFα which negatively impacts CACO-2 epithelial barrier function by downregulation of claudin-1, occludin and ZO-1 at the transcriptional level[197].

Alcohol exposure also negatively affects alveolar epithelial barrier function in rats by decreasing expression of claudins -1 and -7, both known to be important in type I pneumocyte TJs in this animal model[199]. It also decreases claudin-3 expression as well as increasing expression of claudin-5, both known to be changes associated with leakier alveolar epithelia[199-201]. Supplementing alcohol-consumption with zinc acetate decreases alcohol-induced alveolar epithelial cell permeability to sucrose in a dose dependent manner and significantly increases expression and localization of occludin and ZO-1 at aveolar epithelial TJs.

Depleting zinc in alveolar epithelia in the presence of proinflammatory cytokines causes paracellular leak simultaneously with breakdown of E-cadherin and beta-catenin, induction of apoptosis, and activation of caspase-3 activation[202].

It is known that neutrophils can migrate across the TJ during immune response[203] which is initially beneficial, however accumulation of neutrophils causes prolonged inflammation[204]. In zinc-depleted CACO-2 cells, TJ barrier function was disrupted as evidenced by decreased R_t and disruption of occludin and ZO-1 expression (attributed to change in phosphorylation state). F-actin and beta-tubulin disorganization were also seen. This allowed increased transmigration of neutrophils across the cell layer[109].

Chronic Fatigue Syndrome

There is evidence that a leaky gut is a factor in Chronic Fatigue Syndrome (CFS) since endotoxin levels in CFS patients (as measured by increased IgM and IgA serum levels against LPS) are elevated. A case study showed a complete remission of CFS symptoms after being put on a leaky gut diet (low carbohydrate, gluten and milk free), in conjunction with intravenous immunoglobulins (IVIGs) and NAIOs (natural anti-inflammatory and anti-oxidative substances, namely N-acetyl-cysteine, glutamine and zinc, among others) which resulted in normalization of IgM and IgA levels and normalization of LPS translocation[205]. Presumably this was due to a tightening of the intestinal barrier. In a follow up study, CFS patients followed only the leaky gut diet and took NAIOs without the addition of IVIGs, and had similar results, meaning that these two therapies alone tightened leaky TJs in the gut, reducing translocation of endotoxin into the bloodstream[206].

Cadmium toxicity

Cadmium is an environmental hazard found, for example, in fossil fuels and fertilizers, and it can accumulate in the human body causing nephrotoxicity and other morbidities[207]. When exposed to the renal epithelial cell culture, LLC-PK$_1$, cadmium disrupted TJs, resulting in decreased R_t[208]. Zinc significantly inhibits changes to inulin U/P (urinary to plasma ratio), GFR (glomerular filtration rate), and urinary flow rate in cadmium-exposed rats and, given enough time, it can completely inhibit the negative effect of cadmium on renal function. Zinc treatment also protected against cadmium-induced disorganization of claudins -2 and -5 at the TJs in preparations of rat kidney tubules in a time-dependent manner[207].

Zinc likewise exerts a protective function against copper or iron toxicity. This can occur by both induction of metallothioneins as well as simply competition at the same ligand sites[209]. Zinc has been shown to prevent copper- and iron-related cirrhosis in rats[210] as well as iron accumulation in a simpler cell culture model[211].

Malnutrition

Malnutrition is also known to negatively impact intestinal barrier function. Rodriguez *et al*[65] (1996) found that malnourishment causes increased ionic conductance, mannitol and Na^+ permeability of the jejunal epithelium of guinea pigs, but zinc supplementation kept these permeability levels comparable to controls. Additionally, whereas freeze fracture electron microscopy showed malnourished guinea pigs' jejunal epithelia had 10% less TJ strands, this was not the case when malnourished animals were supplemented with zinc (1996). Treatment with zinc appears to alleviate small intestine paracellular leak due to malnutrition and protects TJs from degradation.

Inflammatory bowel diseases

When zinc is enterally delivered to rats with TNBS-induced colitis, the increase in paracellular permeability to Na^+ and mannitol in the distal colon is partially ameliorated[212]. In a related study, rats treated with $ZnSO_4$ prior to TNBS-induced colitis, weighed more, had less diarrhea, and showed increased MT synthesis, but $ZnSO_4$ did not affect inflammation, neutrophil invasion of the colonic mucosa, distension of the colon, or zinc concentrations[136]. Interestingly, rats pre-treated for 3 d with a high dose of zinc (30 mg/kg) before colitis was induced *via* intrarectal DNBS (dinitrobenzene sulfonic acid) showed significantly fewer TJs that were leaky to lanthanum and gained more weight than controls, but a lower dose of zinc did not show this effect (despite still being 10 times the daily requirement for humans)[82]. The level at which zinc conveys the maximal benefit without detrimental effects must be investigated more thoroughly.

In human studies, CD patients who were in remission but displayed increased permeability of the small intestine had a significantly lower lactulose/mannitol ratio when given $ZnSO_4$ supplements. Ten of the 12 patients reached normal permeability levels.

Chemotherapy and NSAIDs

Further studies of zinc's effect on barrier function in specific GI-related disease states have also been promising. When rats with methotrexate (MTX)-induced mucositis (a state mimicking intestinal mucosal injury in chemotherapy) were administered oral zinc alone or in conjunction with bovine whey-derived growth factor extract (WGFE) they showed decreased permeability of their intestine to ^{51}Cr-EDTA[213]. The combination of zinc + WGFE was the most effective in recovery of the intestine post-MTX injury, providing further evidence that zinc can enhance the beneficial properties of other treatments. In a human study examining the effects of the easily available natural food supplement, zinc carnosine (ZnC), on NSAID-induced intestinal leakiness, ZnC protected against the typical increase in small intestine permeability from indomethacin as well as indomethacin-induced gut injury[214].

Infectious disease

Bacterial infection can distinctly target epithelial barrier function. ETEC increases paracellular flux of inulin and

decreases R_t in CACO-2 cells. While ZnO treatment of *healthy* CACO-2 cells did not affect TJ permeability, ZnO treatment of ETEC-infected cells inhibited the ETEC-induced increase in inulin paracellular leak while maintaining R_t comparable to control cell layers in a time and dose-dependent manner[215]. Given that TNFα is known to cause breakdown in epithelial barrier function[216] whereas TGFβ can enhance TJ barrier function[217], it is also of note that TNFα expression was elevated and TGFβ was drastically reduced in ETEC-infected cells. Increasing ZnO concentrations counteracted these effects[215]. It should be noted that this study chose to use ZnO rather than $ZnSO_4$ because ZnO is more effective in skin epithelial wound healing[218]. Further investigation is warranted into the most effective zinc source, keeping in mind that it could be different depending on the tissue and physiology.

Mycotoxins

Ochratoxin A (OTA) is a mycotoxin that is both a food contaminant and potential human carcinogen. Although it takes over 24 h of exposure, OTA negatively affects barrier function in CACO-2/TC7 cell layers by reducing claudins -3 and -4 in TJs and increasing TJ permeability[219,220]. Zinc-depleted CACO-2/TC7 cells exposed to OTA showed increased TJ permeability (as measured by decreased R_t) and increased apoptosis. Zinc depletion significantly enhanced the deleterious effects of OTA on CACO-2/TC7 cells[219].

Hyperthermia

Hyperthermia increases intestinal mucosal permeability[221] and is known to affect the expression of TJ proteins[222,223]. Heat stress is known to negatively affect porcine growth and production during the warmer part of the year. When varying concentrations of zinc amino acid complex (ZnAA) were fed to growing pigs exposed to heat stress (HS), those exposed to ZnAA showed improved intestinal barrier function on day 7, although the lower level of ZnAA (+100 ppm over control levels) showed greater improvement than the higher level (+220 ppm over controls)[224]. This could be because higher concentrations are known to negatively affect the pancreas and other organs[225].

Diarrhea

Post-weaning diarrhea is a common cause of death in piglets and the resulting increase in gut permeability - likely due at least in part to alterations in the TJs - could be the reason[226,227]. Piglets fed either tetrabasic zinc chloride or ZnO showed decreased lactulose/mannitol ratios in their urine as well as increased expression of the TJ proteins occludin and ZO-1 in the ileum[228]. Piglets fed diosmectite-zinc oxide (DS-ZnO) showed comparable results[229]. These studies suggest that diarrhea is improved by zinc specifically *via* alleviation of intestinal barrier compromise[228].

Cell culture studies

In epithelial cell culture studies, zinc's effects on barrier function are promising. Zinc supplementation decreases expression of claudins -2 and -7 in CACO-2 cell layer TJs and improves barrier function to electrolytes (as shown *via* increased R_t), although paracellular permeability to small nonelectrolytes (mannitol) is actually increased[166]. In the renal epithelial cell culture line LLC-PK1 zinc increases R_t and significantly decreases transepithelial mannitol leak. Although changes in claudin levels in whole cell lysates were not seen with zinc supplementation, increases in cytosolic levels of claudins -2 and -4 were observed[166]. A combination of the micronutrients, zinc and quercetin, had an even stronger positive effect on R_t, but still did not significantly affect mannitol leak. The zinc/quercetin combination also seemed to further increase claudin-7 expression in the cells[230]. Apical exposure of the renal epithelial cell line MDCK II to zinc chloride, conveys high R_t to the normally leaky cell line[231]. MDCK cell layer conductance is predominantly paracellular, so the increase in R_t (*i.e.*, decrease in conductance) due to zinc chloride treatment likely involves a paracellular pathway[232]. Claudin-2 confers cation-selective pores in leaky epithelia such as MDCK[233]. In zinc-treated cells, claudin-2 internalization and degradation was increased, quite possibly being the reason for the tighter barrier. It should be noted that application of zinc chloride to both the basal-lateral and apical sides of MDCKII cells (simultaneously) causes a drop in R_t[231]. The barrier properties of the colon epithelial cell line, T84, however, showed no change when incubated in Ussing chambers with zinc for short time periods (30 min or less)[234].

Summary

Zinc supplementation has been shown to have a protective effect on the epithelial barrier - specifically at the level of the TJ - in cell lines, animal models, and humans in a variety of pathologies including chronic alcohol exposure, heat stress, diarrhea, CFS, colitis, other GI ailments, and even some neurological conditions. Often it can enhance the effects of other beneficial molecules, such as WGFE and quercetin. Whereas in a biological system at homeostasis there is evidence that zinc can improve baseline barrier function, zinc can also protect against both pathophysiologically-generated TJ and general barrier leak. Zinc deprivation, on the other hand, can have catastrophic effects and aggravate disease states *via* induced barrier leak. It should be mentioned, however, that one electron microscopy-based study of severe ZD in rats did not show junctional leak (to the electron dense tracer, lanthanum ion)[235]. The optimal level of zinc and the best form for delivery to distinct target tissues still require further investigation, as do the precise mechanisms involved in its regulation of TJs. However, its positive impact on epithelial barrier function is undeniable.

CONCLUSION

The state of ZD is very favorable to the development of various GI diseases, deriving in large part through its negative effect on GI epithelial barrier function. ZD in the United States seems counterintuitive, but with extensive use of PPI drugs, diets abundant in phytate-rich foods, and decreasing consumption of meat and fish in general, lower zinc body stores are not out of the question. Zinc supplementation could be a highly inexpensive and, within well-described daily dosage limits, quite safe, prophylactic measure against several distinct classes of GI disease. Zinc could also serve to possibly reduce the morbidity of certain established diseases. The utility of zinc as an adjuvant to certain current pharmacologic treatments may have real merit. Ironically if one refrains from expecting unrealistic effects of zinc, *i.e.*, out and out cures of certain GI diseases, its real merit and value comes into focus.

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REVIEW

Alterations of the gut microbiome and metabolome in alcoholic liver disease

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Abstract

Alcohol consumption is one of the leading causes of liver diseases and liver-related death worldwide. The gut is a habitat for billions of microorganisms which promotes metabolism and digestion in their symbiotic relationship with the host. Alterations of gut microbiome by alcohol consumption are referred to bacterial overgrowth, release of bacteria-derived products, and/or changed microbiota equilibrium. Alcohol consumption also perturbs the function of gastrointestinal mucosa and elicits a pathophysiological condition. These adverse effects caused by alcohol may ultimately result in a broad change of gastrointestinal luminal metabolites such as bile acids, short chain fatty acids, and branched chain amino acids. Gut microbiota alterations, metabolic changes produced in a dysbiotic intestinal environment, and the host factors are all critical contributors to the development and progression of alcoholic liver disease. This review summarizes recent findings of how alcohol-induced alterations of gut microbiota and metabolome, and discusses the mechanistic link between gastrointestinal dyshomeostasis and alcoholic liver injury.

Key words: Alcoholic liver disease; Microbiome; Gut metabolome

Core tip: Excessive alcohol consumption causes alcoholic liver disease (ALD) with the mechanisms of pathogenesis largely unknown. Alterations of gut microbiota and metabolites are critical contributors to the development of ALD, which may lead to identification of therapeutic targets for ALD. This review summarizes recent findings of how alcohol-induced alterations of gut microbiota and metabolome, and discusses the mechanistic link between gastrointestinal dyshomeostasis and alcoholic liver injury.

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INTRODUCTION

Alcohol abuse is one of the leading causes of liver disease-related morbidity and mortality worldwide. Alcoholic liver disease (ALD) may progress from steatosis (fatty liver) to steatohepatitis, liver cirrhosis, and eventually hepatic carcinoma[1,2]. According to the National Institute on Alcoholic Abuse and Alcoholism, liver cirrhosis is the 12th leading cause of death in the United States, about 50% of which are alcohol related[3]. Even though enormous efforts have been made, the pathogenesis of ALD is still poorly understood, which makes the progress in finding proper treatments slow. In the last decade, the

role of the gastrointestinal tract (GI) in the pathogenesis and progression of ALD has drawn more and more attention. It is estimated that there are multiple times more microbial cells in the gut than the total number of cells in the human body[4]. The microbes contribute to a complex of biological processes such as digestion[5], synthesis of vitamins[6], and regulation of immunity[7]. Disruption of intestinal homeostasis and alterations in the gut microbiome and metabolome contribute to the pathogenesis of many disorders including ALD[4,8,9]. This review summarizes recent findings on how alcohol affects the composition of the gut microbiota and metabolites, and discusses the mechanistic link between GI dyshomeostasis and the pathogenesis of alcohol consumption-induced liver injury.

INTESTINAL MICROBIOME AND ALCOHOLIC LIVER DISEASE

Quantitative (bacterial overgrowth) and qualitative (dysbiosis) changes of the GI microbiome have long been associated with liver diseases including ALD[10]. Disturbed gut microbiota homeostasis results in dysfunction of the intestinal barrier and translocation of bacteria and/or bacterial products, which eventually contribute to the progression of ALD. Interventions focusing on gut bacteria and/or bacterial products in preventing ALD have drawn increasing attention in the last decade.

Intestinal bacterial overgrowth and translocation in the development of ALD

Alcohol consumption is well known to elicit bacterial overgrowth along the GI tract[11]. The number of both aerobic and anaerobic bacteria cultures of jejunal aspirates from alcoholic patients was distinctly higher than that from the control patients[12]. Similar trends were observed in patients with alcoholic cirrhosis[13]. Bacterial overgrowth has also been documented in experimental models of ALD[14,15]. Overgrowth of bacteria affects ethanol metabolism. Experimental induction of bacteria overgrowth resulted in enhanced endogenous and/or exogenous ethanol metabolism and high concentrations of acetaldehyde in both the intestinal lumen and the portal blood[16-18]. Oral administration of metronidazole, an antibiotic drug, led to a higher level of intracolonic acetaldehyde by increasing aerobic bacteria and reducing anaerobic bacteria in the intestine[19]. On the other hand, intracolonic acetaldehyde accumulation was prevented by antibiotic ciprofloxacin, which decreased colonic microbiota and fecal alcohol dehydrogenase activity[20].

Bacterial translocation is defined as the passage of viable bacteria from the GI tract to extraintestinal sites, such as the mesenteric lymph node, liver, kidney, and bloodstream. Experimental induction of small bowel bacterial overgrowth caused bacterial translocation in association with hepatic inflammation in rats[21]. The translocation of bacteria has been reported as early as 14 d after alcohol consumption in rats[22], while some studies did not show significant bacterial translocation after alcohol administration for 2 wk[23,24]. Moreover, Yan *et al*[14] reported that the bacterial translocation occurred prior to changes observed in the microbiome in a mouse model of continuous intragastric alcohol feeding for up to 3 wk. On the contrary, in a rat model of ALD combined with bacterial inoculation, rats chronically fed with alcohol presented markedly less bacterial translocation to the mesenteric lymph nodes and to the other organs examined compared to rats fed with an isocaloric liquid diet[25].

Bacterial products and gut permeability in the development of ALD

Bacteria, particularly the Gram-negative bacteria, produce endotoxins in the GI tract. Under physiological condition, endotoxin is excluded out of the body along with feces, and only trace amount of endotoxin can penetrate through the GI epithelium to the systemic circulation due to the gut barrier[26]. Alcohol consumption increases the serum endotoxin level, namely endotoxemia. The development of endotoxemia mainly results from bacterial overgrowth and/or increased gut permeability. Endotoxemia has been well documented in patients with ALD[26], and the blood endotoxin levels correlate well with tumor necrosis factor α (TNF-α) levels and the severity of ALD[27-29]. Elevated endotoxin in systemic circulation activates hepatic Kupffer cells *via* Toll-like receptor 4 to produce inflammatory cytokines and chemokines which, in turn, attract neutrophils and monocytes into the liver[30]. In addition to endotoxin, other bacterial products, such as bacterial DNA, peptidoglycan, and flagellin, could also translocate from the intestinal lumen to extraintestinal space and organs, and play a critical role in ALD progression. It was reported that bacterial DNA was elevated in the plasma of patients with alcoholic cirrhosis[31]. Bacterial DNA is recognized by TLR9 and sensitizes the liver to endotoxin-induced injury[32]. Alcohol exposure increased peptidoglycan levels and injected peptidoglycan deteriorated liver injury and inflammation in alcohol-fed mice[33,34].

Intestinal barrier dysfunction has been repeatedly reported in alcohol-induced endotoxemia and liver damage. Alcoholic patients showed increased gut permeability to a variety of macromolecules, such as polyethyleneglycol, lactulose/mannitol, or ^{51}CrEDTA[35-38]. In animal studies, gut permeability to macromolecules such horse radish peroxidase was also increased in association with alcohol-induced endotoxemia and liver damage[39-43]. Orally administrated lipopolysaccharide could be detected in the plasma of acute alcohol-intoxicated mice but not in the control mice[44]. Chronic alcohol exposure reduced the distribution of tight junction proteins, but did not significantly affect the intestinal histopathology[45]; and the gut leakiness only occurred in the ileum instead of in the duodenum or jejunum[45]. Taken together, intestinal barrier dysfunction enables bacteria and bacterial products to translocate from the intestinal lumen to the liver which, as a result, facilitates the development of ALD.

Intestinal dysbiosis in the progression of ALD

Alcohol consumption not only results in quantitative changes of the intestinal microbiota, but also leads to enteric dysbiosis. Enteric dysbiosis refers to an imbalance in the intestinal bacterial composition that participates in the normal activities of the GI tract. Clinical studies have shown that patients with alcoholic cirrhosis had a lower proportion of *Bacteroidetes* and higher ones of *Proteobacteria* in the colon as compared to alcoholic patients without liver cirrhosis[46]. In another study, patients with alcoholic liver cirrhosis showed higher amounts of *Prevotellaceae* in the feces compared to cirrhotic patients with hepatitis B or healthy controls[47]. Animal studies also demonstrated that alcohol consumption for 10 wk altered colonic mucosa-associated microbiota composition in rats[48]. The abundance of *Bacteroidetes* and *Verrucomicrobia* were elevated in the cecum of mice intragastrically fed alcohol for 3 wk, while *Firmicutes* bacteria (including *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Lactococcus*) were predominant in the control mice[14]. A recent animal study showed that chronic alcohol feeding for 8 wk caused a decline in the abundance of both *Bacteroidetes* and *Firmicutes* phyla, with a proportional increase in the Gram-negative *Proteobacteria* and Gram-positive *Actinobacteria* phyla in mice feces[15].

The interactions between alcohol-induced liver injury and alterations in the amount/proportion of certain bacteria phylum remain largely unknown. The *Proteobacteria* phylum includes Gram-negative bacteria, most of which are regarded as pathogenic species. Alcohol exposure-induced *Proteobacteria* expansion in the GI tract strongly indicates a link between alcohol-induced alterations of gut microbiota and the elevated plasma endotoxin level as well as hepatic inflammation. Studies have described opportunistic infections of *Corynebacterium*, a member of the *Actinobacteria* phylum, in individuals with ALD[49,50]. As mentioned above, intestinal bacteria like *Escherichia coli* metabolize alcohol and increase luminal acetaldehyde levels through alcohol dehydrogenase-dependent[51] or catalase-dependent pathway[52]. Acetaldehyde is known to disrupt the intestinal barrier through disassembling tight junction proteins[53-56], which implicates another mechanism of how microbiota participate in the development of ALD. The relevance of the gut microbiota changes for ALD progression still requires further investigation.

Intervention for ALD via modulating intestinal microbiome

Efforts on exploring therapeutic strategies for treating ALD have been made for decades, and one of the major attempts was to ameliorate alcohol-induced endotoxemia. Indeed, animal studies demonstrated that abrogating endotoxin signal cascade in the liver by administration of antibiotics[57] or neutralization of circulating endotoxin[58], led to attenuation of alcohol-induced cytokine production and liver damage. Dietary supplementation of milk osteopontin prevented alcohol-induced liver injury through blocking enteric Gram-negative bacterial translocation and the endotoxin-mediated effects in the liver[59].

The effects of probiotics and prebiotics in modulating alcohol-induced liver injury in both patients with ALD and experimental models have been widely studied and the related references are summarized in Table 1. The first report was the study by Nanji *et al*[60], which showed that *Lactobacillus GG* treatment reduced endotoxemia and the severity of ALD. Treatment with *Lactobacillus GG* attenuated alcohol-induced intestinal barrier stress, gut leakiness, and liver injury in rats[40,61-64] and mice[65,66]. A short-term therapy with *Bifidobacterium bifidum* and *Lactobacillus plantarum 8PA3* to alcoholic patients lowered plasma alanine aminotransferase and aspartate aminotransferase levels, restored the gut microbiota, and improved ALD compared to patients treated with standard therapy (abstinence plus vitamins) alone[67]. Another human study showed that *Lactobacillus casei* Shirota administration for 4 wk restored neutrophil phagocytic capacity in alcoholic cirrhotic patients[68]. Notably, the beneficial effects of probiotics were achieved not only by live bacteria, but also by heat-inactivated bacteria[63,69] or bacteria culture supernatant[70].

Short-chain fructooligosaccharides and other prebiotics are used to stimulate the growth and activity of probiotics such as *Lactobacillii* and *Bifidobacteria*. Dietary supplementation of oats prevented alcohol-altered colonic musoca-associated microbiota composition in rats[48]. It was shown that administration of prebiotic (fructooligosaccharides) to alcohol-fed mice reduced bacterial overgrowth and ameliorated alcoholic steatohepatitis through partially restoring the host antimicrobioal protein Reg3g[14].

There are few reports addressing the impact of probiotic and/or prebiotic supplementation on gut microbiome during the development of ALD. To date the most comprehensive study employed 16S ribosome RNA sequencing to characterize gut microbiome changes in mice feces after chronic alcohol exposure and *Lactobacillus GG* supplementation[15]. *Lactobacillus GG* not only reduced bacterial overgrowth in alcohol-fed mice, but also prevented the alcohol-induced expansion of the *Proteobacteria* and *Actinobacteria* phyla.

INTESTINAL METABOLOME AND ALD

Research into alterations in gut metabolome in ALD is unfortunately not as advanced as that for alterations in gut microbiota. To the best of our knowledge, our group, for the first time, applied mass spectrometry-based high throughput technology for characterization of the metabolic alterations of the GI tract contents in a rat model of chronic alcohol consumption. First of all, we conducted a comprehensive metabolite profiling using a high performance liquid chromatography time-of-flight mass spectrometry (HPLC-TOF MS). Secondly, since the HPLC-TOF MS-based profiling approach may not be able to detect or generate accurate data of short chain amino acids (SCFAs) and branched chain

Table 1 Summary of references related to the protective effects of probiotic/prebiotic against alcoholic liver disease

Probiotic/prebiotic	Subjects	Duration of treatment	Outcome	Ref.	Year
Probiotics					
Lactobacillus rhamnosus GG	Male Wistar rats	1 mo	Probiotic feeding reduced alcohol-induced endotoxemia and liver injury	Nanji *et al*[60]	1994
A mixture containing 450 billion bacteria (VSL #3)	Alcoholic cirrhosis patients	3 mo	Treatment of probiotic lowered plasma levels of cytokines and oxidative stress parameters	Loguercio *et al*[103]	2005
L. casei Shirota	Alcoholic cirrhosis patients	4 wk	Probiotic supplementation restored neutrophil phagocytic capacity	Stadlbauer *et al*[68]	2008
Heat-killed *L. brevis* SBC8803	C57BL/6N mice	35 d	*L. brevis* SBC8803 ameliorated alcohol-induced liver injury and fatty liver	Segawa *et al*[69]	2008
Bifidobacterium bifidum and *L. plantarum* 8PA3	Male Russian adults	5 d	Patients treated with probiotics had significantly lower ALT and AST activity, and restored gut microbiota compared to patients treated with standard therapy alone	Kirpich *et al*[67]	2008
L. rhamnosus GG	Male Sprague-Dawley rats	10 wk	*L.* GG reduced alcohol-induced gut leakiness and blunted alcohol-induced oxidative stress and inflammation both in the intestine and liver	Forsyth *et al*[40]	2009
L. rhamnosus GG	Male C57BL/6N mice	Last 2 wk of the 8-wk feeding	*L.* GG supplementation reduced alcohol-induced endotoxemia and hepatic steatosis	Wang *et al*[65,66]	2011, 2013
L.paracasei	Male Fischer 344 rats	10 wk	*L. paracasei* altered the fatty acid composition of the plasma and liver	Komatsuzaki *et al*[61]	2012
L. rhamnosus GG culture supernatant	Male C57BL/6N mice	5 d	Bacteria-free *L.* GG culture supernatant ameliorated acute alcohol-induced gut leakiness and liver injury	Wang *et al*[70]	2012
Combined *Bifidobacterium*, *Lactobacillus*, *Enterococcus* and *Bacillus cereus* tablets	Male Sprague-Dawley rats	Up to 8 wk	Probiotic administration reduced plasma elevated-endotoxin levels caused by alcohol and altered gut microbiota	Zhang *et al*[62]	2012
Live or heat-killed VSL #3	Male rats	Up to 12 h	VSL #3 administration reduced plasma endotoxin level and cytokine production caused by acute alcohol exposure	Chang *et al*[63]	2013
Heat-killed *L. casei* MYL01	HepG2 cells	20 h	*L. casei* MYL01 modulated proinflammatory cytokine production	Chiu *et al*[104]	2014
Escherichia coli Nissle 1917 secreting pyrroloquinoline quinone	Male Foster rats	10 wk	Probiotic treatment ameliorated alcohol-induced oxidative damage and hyperlipidemia in rats	Singh *et al*[64]	2014
Prebiotics					
L. rhamnosus GG or oats	Male Sprague-Dawley rats	10 wk	Supplementation of *L. rhamnosus* GG or oats prevented alcohol-altered colonic musoca-associated microbiota composition in rats	Mutlu *et al*[48]	2009
Fructooligosaccharides	Male C57BL/6J mice	3 wk	Administration of fructooligosaccharides to alcohol-fed mice reduced bacterial overgrowth and ameliorated alcoholic steatohepatitis through partially restoring the host antimicrobial protein Reg3g	Yan *et al*[14]	2011

fatty acids (BCAAs) due to their volatile properties, a gas chromatography mass spectrometry (GC-MS) was used to quantitatively measure specific metabolic panels of SCFAs and BCAAs. The methods were described in more detail elsewhere[71,72]. Thirdly, a targeted quantitative metabolomics approach for a panel of 20-30 bile acids using ultraperformance liquid chromatography-triple-quadrupole mass spectrometry was utilized[73]. Alcohol consumption markedly altered bile acids[73], increased fatty acids and steroids, decreased carnitines, amino acids, branched chain amino acids, and all short chain fatty acids except for acetic acid[71] in the GI luminal contents of rats after 8-wk of alcohol exposure. Bile acids, SCFAs, and BCAAs were the top three categories among the significantly changed metabolites by alcohol consumption. Therefore, they were quantitatively measured in our study and the results will be discussed in more detail below.

Global profiling of metabolites in the GI tract

Chronic alcohol consumption resulted in a global metabolite alteration including amino acids, fatty acids, steroids, lipids, carnitine, SCFAs, BCAAs[71], and bile acids[73] along the GI tract of rats. Almost all amino acids detected were decreased in GI contents of alcohol-fed rats compared to the control. Notably, high abundances of alanine, arginine, glutamic acid, proline, and threoine were observed in all the intestinal segments (from duodenum to rectum) and they were dramatically decreased after alcohol exposure. Amino acids derived from dietary protein may serve as substrates for luminal conversion by the gut microbiota which, in turn, regulate the host homeostasis. For example, one constituent of the gut microbiome, *Lactobacillus reuteri*, is able to convert L-histidine into histamine, which is an immune-regulatory signal suppressing TNF-α production[74]. Intestinal bacteria also involve in converting glutamate to γ-amino butyric acid *via* gluta-

mate decarboxylase[75]. Taken together, it is possible that the reduced abundance of amino acids in alcohol-fed rats was resulted from a perturbed gut microbial-host co-metabolism under the enteric dysbiosis condition.

The levels of steroids and steroid derivatives were significantly increased after alcohol consumption in the stomach, duodenum, jejunum, and ileum. Carnitines and metabolites involved in lipid metabolism were decreased in alcohol-fed rats. Most of the fatty acids detected were at higher levels including 17-HDoHE and 19,20-DiH-DPA, the two metabolic products from docosahexaenoic acid (DHA), and DHA itself. The elevation of DHA and DHA metabolites in the intestinal lumen, especially the large intestine, indicates a disrupted absorption of this nutrient induced by alcohol exposure.

Bile acids

Alcohol consumption significantly perturbed all 21 bile acids detected along the GI tract with the ileum showed the most significant alteration[73]. The concentration of unconjugated bile acids in control rats was low in duodenum (0.04 nmol/mg wet weight), whereas it was increased in the alcohol group (1.30 nmol/mg wet weight). Taurine-conjugated bile acids are the most abundant bile acids in the small intestine and the liver of control rats[73,76,77]. Alcohol consumption led to lower levels of taurine-conjugated bile acids in the duodenum and ileum (0.15 and 0.02 nmol/mg wet weight) compared to control rats (2.39 and 5.66 nmol/mg wet weight, respectively), which made unconjugated bile acids accounted for the largest proportion of the total bile acids in the entire GI tract. Meanwhile, the amount and proportion of taurine-conjugated bile acids were decreased both in the liver and blood[73].

Bile acid metabolism is dependent on the biological activities of the gut microbiota and the host, and both bacterial and hepatic enzymes further modify bile acids during enterohepatic circulation[78,79]. Perturbed gut microbiome may result in a disturbance of bile acid metabolism and reabsorption, leading to altered bile acid profiles in the blood, liver, kidney, and heart[80]. Indeed, inhibiting intestinal microbiota with ampicillin increased the expression of the apical sodium-dependent bile acid transporter (ASBT/Slc10a2) in the brush-border membrane of the ileum, which in turn increased bile acid transport into portal blood[81]. Germ-free mice and rats have a higher proportion of taurine-conjugated bile acids in their livers and intestines[79,82], demonstrating a close association between gut microbiota and bile acid composition. It has been reported that the ratio of glycine-conjugated to taurine-conjugated bile acids is dependent on the hepatic taurine concentration[83]. In our study, we found that the hepatic bile salt taurine to glycine ratio was 30:1 in control rats, while the ratio was 1:1 in alcohol-treated rats. The majority of taurine is usually degraded by the gut microbiota to inorganic sulfate[84]. For this reason, an overgrowth of gut microbiota caused by alcohol exposure would be expected to decrease taurine bioavailability, which provides an explanation for alcohol-induced decrease in taurine-conjugated bile acids in our study. In addition, another investigation suggests that the reduction of taurine in the liver in alcohol-fed mice may be due to the formation and excretion of N-acetyltaurine, a novel metabolite synthesized from taurine and acetate[85].

SCFAs and BCAAs

Acetic acid, propionic acid, and butyric acid are the most predominant SCFAs within the intestine[86]. Our study revealed that the distal intestine (ileum to rectum, especially cecum) processed the majority of SCFAs, within which acetic acid, propionic acid, and butyric acid were predominant (85% in ileum, 94% in cecum, 97% in colon, and 93% in rectum)[71]. Alcohol consumption dramatically reduced all 9 SCFAs detected in the distal intestine except for acetic acid. SCFAs are mainly produced by microbial fermentation of indigestible dietary fibers in the gut[87]. The alteration of SCFAs in alcohol-fed rats may be a result from alcohol-perturbed gut microbiota. The elevated acetic acid levels after alcohol consumption may presumably be due to the oxidation of ethanol to acetaldehyde and subsequent oxidation to acetic acid[88]. Since bacterial aldehyde dehydrogenase activity is limited[18], gut microbiota may not be the major player for the elevated luminal acetic acid level. On the other hand, SCFAs may influence the gut microbiota through stimulating *Bifidibacteria* growth and inhibiting Gram-negative facultative and anaerobic bacteria[89]. SCFAs are known as energy sources to regulate the homeostasis of the intestine and other organs[86]. In a recent study, SCFAs were approved to be beneficial against alcohol-induced intestinal barrier dysfunction through activating AMP-activated protein kinase in Caco-2 cells[90].

BCAAs are essential nutrients obtained from food, as they cannot be synthesized *de novo* by mammals[91]. Gut microbiota, however, are capable to produce BCAAs efficiently[92]. BCAA supplementation has been widely used to improve energy metabolism[93,94], insulin resistance[95-97], and severity of liver disease[98]. Our study reported that all three BCAAs, valine, leucine, and isoleucine, in the GI lumen were predominant in the small intestine (duodenum, jejunum, and ileum) and to a lesser extent in the cecum in rats[71]. Alcohol consumption led to significantly lower levels of all three BCAAs in the GI contents[71]. Previous findings have shown that chronic alcohol consumption increased incorporation of leucine into hepatic proteins[99] and accelerated the absorption of leucine from the small intestine[100], which may explain the dramatic reduction of BCAAs in the gut lumen observed in our study. Notably, a low ratio of plasma BCAAs to aromatic amino acids is a hallmark of liver cirrhosis. Indeed, elevated leucine and isoleucine levels were reported in the plasma of non-alcholic steatotic and non-alcoholic steatohepatitic patients compared to healthy controls[101], which indicate the homeostasis of BCAAs may be involved in the pathogenesis of liver diseases. Moreover, branched chain SCFAs, 2-methylpropanoic acid, 2-methylbutyric acid, and 3-methylbutyric acid are derived from the catabolism of BCAAs[102]. The decreased enteric BCAA levels may

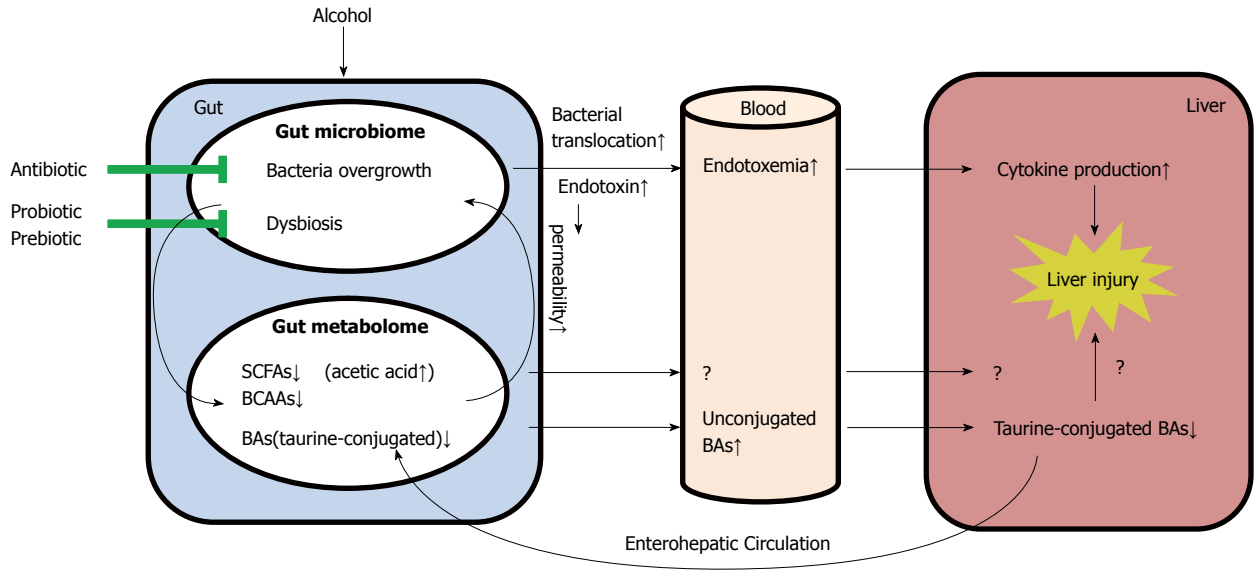

Figure 1 Schematic diagram of the impact of alcohol consumption on the gut microbiota and metabolome during the development and progression of alcoholic liver disease. BA: Bile acids; SCFA: Short chain fatty acid; BCAA: Branched chain amino acid.

further contribute to the decreased levels of branched chain SCFAs after alcohol consumption.

CONCLUSION

Alcoholic consumption is one of the leading causes of liver diseases and liver-related death worldwide. Of the major factors that contribute to the pathogenesis of ALD, the gut microbiota and metabolites have recently drawn more and more attention. Altered intestinal microbiota and gut-associated endotoxemia are recognized as pathophysiological factors in the development of ALD. Prebiotics and probiotics have been applied to prevent alcohol-induced disease development and progression. Taking the advantages of metabonomics approaches, detailed metabolic profiling provides novel information on alcohol-induced alterations in microbiota-host co-metabolism. The impact of alcohol consumption on the gut microbiome and metabolome during the development of ALD is summarized in Figure 1. Despite the recent progression in understanding the importance of the GI tract in the development of ALD, questions of how alcohol consumption results in gut microbiome and metabolome alterations and what are the consequences of such changes to the host have not been fully addressed. Future investigations on the cause-effect relationship between alterations of gut microbiome/metabolome and the liver pathophysiology will not only provide novel insights into the pathogenesis of ALD but also pave the way to the development of therapeutic interventions to cure ALD.

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REVIEW

Continuing challenges in the diagnosis and management of obscure gastrointestinal bleeding

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Abstract

The diagnosis and management of obscure gastrointestinal bleeding (OGIB) have changed dramatically since the introduction of video capsule endoscopy (VCE) followed by deep enteroscopy and other imaging technologies in the last decade. Significant advances have been made, yet there remains room for improvement in our diagnostic yield and treatment capabilities for recurrent OGIB. In this review, we will summarize the latest technologies for the diagnosis of OGIB, limitations of VCE, technological enhancement in VCE, and different management options for OGIB.

Key words: Obscure gastrointestinal bleeding; Video capsule endoscopy; Deep enteroscopy; Computed tomography enterography; Magnetic resonance enterography

Core tip: Since the advent of capsule endoscopy, significant advances have been made in the imaging of the small bowel that allow for the diagnosis and management of obscure gastrointestinal bleeding. With a decade of knowledge, it is now appropriate for us to look back, critically evaluate our achievements, improve on our current technologies and develop ideas to circumvent some of the shortcomings.

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INTRODUCTION

Remarkable progress has been made since 2001 in the development of technologies that are available to investigate disorders of the small intestine. The wireless video capsule endoscope (VCE) and double balloon enteroscope became available in 2001, followed by the development of computed tomography enterography (CTE) and magnetic resonance enterography (MRE). These tools and newer variations have allowed us to diagnose and manage small bowel lesions in ways that were previously unimaginable. After a 10 year period of remarkable progress it is appropriate to look back, critically evaluate where we are, and use that evaluation as a springboard to critique the technologies and circumvent some of their shortcomings.

Many studies have shown that the diagnostic yield of VCE and deep enteroscopy are similar for obscure gastrointestinal bleeding (OGIB) - approximately in the 40% to 60% range. The higher numbers are for overt obscure bleeding (40% to 92%), occult bleeding (40% to 60%), and iron deficiency (10% to 30%). Assuming indications for these studies are appropriate, a failure-to-diagnose rate of 50% with current techniques appears to be an inconvenient truth. Similarly, recurrent bleeding is distress-

Table 1 Specifications of available capsules

	MiroCam (IntroMedic)	PillCam SB/SB2/SB3 (Given Imaging)	EndoCapsule, EC-10 (Olympus)
Size (mm)	11 × 24	11 × 26	11 × 26
Weight (g)	3.4	3.45	-
Resolution (pixels)	320 × 320	256 × 256	-
Frames per second (fps)	3	2	2
Battery life (h)	11	8-12	8-12
Field of view (°)	150	140/156	145
Communication	Human body communication	Radiofrequency	Radiofrequency
Real time viewer	Yes	Yes	Yes

ingly common. A recent large study from South Korea involving 13 centers and 305 patients showed a detection rate of 51.5%. After VCE only 11.8% received interventional treatment. The overall re-bleeding rate was 19% during the mean of 30 mo[1]. Interestingly the re-bleeding rate was not different between those with positive capsule results and those that had therapy. These observations confirm that we still have a long way to go in terms of better diagnosis and therapy.

DIAGNOSIS OF OGIB

Technology

Various invasive and non-invasive modalities are available for the evaluation of OGIB. These include VCE, deep enteroscopy and a variety of radiological modalities such as CTE, MRE, and conventional and provocative angiography.

Video capsule endoscopy: Currently there are three FDA video capsule endoscopes available in the United States (Table 1). PillCam SB2 (now SB3; Given Imaging, Yoqneam, Israel), EndoCapsule (now EC-10; Olympus, Tokyo, Japan), and MiroCam (IntroMedic, Seoul, South Korea). These capsules are all approximately the same size (11 × 24-26 mm). Most are able to image for up to 12 h, thus reducing the occurrence of incomplete transit. The field of view for all is also similar (140°-156°).

CapsoCam (Capsovision, Saratoga CA, United States) which is in clinical trials in the United States has four cameras allowing for 360° imaging. Unlike traditional capsules that take pictures at a rate of 2-3 frames per second (fps), each of the CapsoCam camera images at the rate of 5 fps for the first two hours and thereafter at 3 fps resulting in 20 and 12 fps respectively. In a prospective comparative study by Pioche *et al*[2], both CapsoCam and PillCam SB2 were found to have similar diagnostic yield (81.8% and 84.8% respectively), however, CapsoCam detected significantly more lesions (108 lesions *vs* 85 lesions), but had a longer reading time (32.0 min *vs* 26.2 min)[2].

MiroCam uses Human Body Communication (HBC) for data transmission which is different from the radiofrequency telemetry of PillCam SB2, SB3 and EndoCapsule. HBC technology uses the human body as a conductive medium for data transmission which expends less electrical power, thereby conferring a longer operating time (12 h) and providing a higher resolution of images (320 × 320 pixels) compared to PillCam SB2 (256 × 256 pixels)[3]. Complete small bowel examination was achieved in 93.3% for MiroCam compared with 84.3% for PillCam SB2. When comparing both capsules for the evaluation of OGIB, the overall concordance was 78.65%, with 77.42% positive agreement and 79.31% negative agreement. MiroCam also has a 42% reduction in missed lesions compared with PillCam[4]. However, the longer recording time along with more image frames captured per second also translate to longer reading time, which may negate the 9% higher rate of small bowel completion and lower missed lesion rate with MiroCam.

Overall, the diagnostic yield of VCE is reported as 38%-92%[5-7]. With the use of VCE in the evaluation and management of OGIB, rebleeding rate was noted to be 15.6% within a 12 mo follow-up period[8]. VCE has the highest yield (92.3%) in those with active overt gastrointestinal (GI) bleeding, and the lowest diagnostic yield (12.9%) in those with a history of obscure GI bleeding[9,10].

Deep enteroscopy: Deep enteroscopy can be performed using double balloon enteroscopy (DBE), single balloon enteroscopy (SBE) or through spiral enteroscopy (SE). These techniques work by pleating the small bowel back over the overtube to minimize looping, thereby allowing the enteroscope to advance forward.

DBE involves two balloons, one each on the distal end of the enteroscope and the overtube. Studies have found the diagnostic yield of DBE is approximately 60%-80% for evaluating OGIB. Success rate of total enteroscopy, with the evaluation of the entire small bowel, either in the antegrade or antegrade plus retrograde fashion, was reported to be 16%-86%[11].

Kamalaporn *et al*[12] looked at detection rates of combined DBE following VCE in OGIB. Overall detection rates for both techniques were similar. Each technique detected lesions not seen by the other, and are complementary in the evaluation of OGIB. VCE is generally performed before DBE, as it can potentially localize the bleeding source and guide the direction of subsequent deep enteroscopy, either in the antegrade or retrograde fashion[13]. In a recent systematic review of over 12000 DBE over a 10 year period, the most common indication for performing DBE (62.5%) was for management of suspected bleeding in the small bowel. DBE was successful in detecting small bowel bleeding in 68.1%[14].

In contrast, SBE involves one balloon. With the use of an angulated enteroscopic tip that can hook onto the small bowel, this technique allows for the enteroscope to advance with a single balloon. Diagnostic yield and intervention rate of SBE were similar to that of DBE (57% *vs* 53% and 32% *vs* 26% respectively)[15], and the procedure

times were both the same at 60 min[16].

SE consists of the Endo-Ease Discovery SB which is a 118 cm long spiral-shaped overtube with spiral ribbing on its surface that is used for enteroscopy *via* the oral route. Diagnostic yield of SE was 57.1%, similar to that of SBE and DBE, and 60% of the angioectasias seen on VCE were detected during SE[17]. Recurrent overt bleeding after SE was 26% during a mean follow up of 2 years[18]. Compared with DBE, SE had both a shorter examination time, along with shorter time to reach the farthest point of examination (43 min *vs* 65 min and 24 min *vs* 43 min respectively). However, DBE allowed for deeper advancement of the enteroscope than SE (310 min *vs* 250 cm)[19].

A recent comparison review noted similar diagnostic yields for SBE, DBE, and SE (53.9%, 64.4%, and 47.0% respectively). Procedure time was fastest in the SE group (oral: 41.0 min, anal: 46 min) followed by SBE (oral: 59.8 min, anal: 68.8 min), and DBE (oral: 71.6 min, anal: 84.5 min). Therapeutic interventions were highest for DBE (40.1%), compared with SBE and SE (26.8% and 29.7%)[20].

CTE and MRE: Similar to VCE, CTE may be used in the evaluation of OGIB to provide a potential road map prior to performing the more invasive DBE. In comparing CTE and VCE, the diagnostic yield for all findings was 34% and 53% respectively. This yield was similar for the detection of neoplastic or mass lesions. However, VCE was superior over CTE in the detection of vascular or inflammatory mucosal lesions. In comparing CTE with DBE, the diagnostic yield was higher for DBE (78% *vs* 38%). Other studies comparing CTE with digital subtraction angiography showed similar yield of 64% and 60% respectively[21].

Agrawal *et al*[22] recently reported that in patients which VCE failed to localize bleeding, CTE may have a utility for the subsequent work up of overt, but not occult or OGIB. The authors found the diagnostic yield to be 50% in overt GI bleed, but 0% in OGIB.

MRE may be considered as an alternative for the initial examination in patients with clinical suspicion of small-bowel stenosis[23] and is also used as a complementary modality for evaluation of patients with small bowel tumors and Crohn's disease.

Angiography: Limited data exists on provocative angiography. In a single center series of patients with obscure and recurrent lower GI bleed, Kim *et al*[24] reported successful definitive treatment of recurrent hemorrhage in 11 of 36 studies (31%) that were performed on 34 patients, with only one complication of ischemic bowel perforation that necessitated bowel resection. There were otherwise no bleeding complications. Leung *et al*[25] compared VCE and angiography for acute overt OGIB and found that the diagnostic yield of VCE was significantly higher than that of angiography (53.3% *vs* 20.0%).

LIMITATIONS OF CAPSULE ENDOSCOPY

Why do we miss lesions?

It has been reported that VCE missed about 11% of all abnormalities in the small bowel. With single mass lesions, the miss rate can be up to 18.9%[26]. Multiple factors account for missed lesions in VCE, including rapid transit through the duodenum and proximal jejunum, unidirectional field of view (about 150°), coupled with a non-axial transit which does not permit the camera to capture the entirety of the mucosal surface. This latter issue explains the discordance of repeat capsule studies in the same patient. Furthermore, inadequate luminal distension and the presence of luminal contents and bubbles further impair complete visualization of the mucosa.

Despite the ability of deep enteroscopes to distend the intestine and wash the mucosa, the diagnostic yield is comparable to that of VCE in most patients when the first deep enteroscopy is attempted from only one direction[12]; achievement of pan enteroscopy is uncommon. The field of view for deep enteroscopes is comparable to VCE, thus it may be difficult to see lesions on the distal aspect of a fold. Anesthesia may also alter hemodynamics of intestinal blood flow in a manner quite different to that of video capsule, and active bleeding originating from a submucosal source is not commonly seen.

Preparation for capsule endoscopy

Capsule image quality plays an important role in the accuracy of capsule interpretation. Presence of food residue, bile, and air bubbles can obscure images. There are currently no guidelines regarding bowel preparation before VCE. Different centers and studies have used various regimens including overnight fasting only for 12 h, clear liquid diet of varying duration, and use of polyethylene glycol (PEG) and/or simethicone. Studies evaluating the role of purgatives/prokinetics as bowel preparation for VCE have been heterogeneous. In a meta-analysis by Song *et al*[27] and the Korean Gut Image Study Group, small bowel visualization quality was found to be enhanced fourfold with use of bowel prep with PEG solution. Two liter (2 L) of PEG solution was similarly effective as 4 L. Diagnostic yield was also slightly improved using PEG solution as compared with overnight fast or clear liquid diet. In another meta-analysis of eight randomized controlled trials comparing use of laxative bowel preparation with fasting alone, PEG based regimens were found to offer better visibility than fasting alone[28]. This is similar to the European Society of gastrointestinal Endoscopy guideline in 2009 recommending purgative bowel preparations that would enhance diagnostic yield of VCE[29]. Use of simethicone with or without bowel prep may also enhance image quality.

Capsule completion rate, however, was not affected with the use of bowel prep, simethicone, or prokinetics. Currently overnight fasting is the standard preparation for VCE in many centers. Alternatively the ASGE recommends 2 L of PEG the evening before the procedure.

Surrounding the controversy regarding use of bowel prep is also the subjective nature of assessing the cleanliness grading system. Current grading systems such as the 10-point quantitative index, or overall assessment of adequacy (adequate or inadequate) are either too cumbersome to calculate or too simplified to be of much utility. With the goal of having an objective assessment, Van Weyenberg *et al*[30] designed a scoring system to assess the quality of bowel preparation with a computed quantitative scale using the color intensities of the tissue color bar. This scoring system, known by the authors as Computed Assessment of Cleansing score is an objective measure, eliminating subjective interpretation by individual readers, and is potentially more reproducible and objective.

Reader error

Recently, a portrayal of physician performance and error in capsule reading was shown in a study by Zheng *et al*[31] to play a big role in missed lesions. In this study, 24 prepared clips of capsule images were read at different modes (single view, duo view, or quad view) and frame speed (15, 20, or 25) by 17 endoscopists, ranging from novice to experienced readers. The detection rates in this study were disappointingly low, ranging from 16% for the detection of blood to 69% for the detection of angioectasias. As expected, ulcers or erosions were more readily detected if they were larger, and masses or polyps were more distinguishable if their color and texture differ from the surrounding mucosa. Abnormal findings appearing in more frames were more likely to be picked up than those appearing in only 1-2 frames. The overall detection rate was also significantly higher when reading in the single view-15 and quad view-20 modes (45% and 47% respectively) compared with reading in single view-25 (26%). This may be explained by the longer dwell time on the screen for each image in quad compared with single view. In another study looking at inter-observer agreement in describing VCE findings, the best agreement was observed in identifying the presence of active bleeding, whereas the worst agreement was in describing size of lesion. Diagnostic concordance was better with angioectasias than for polyps or ulcers/erosions[32].

Missed lesions in the proximal small intestine

Several case reports have noted missed lesions on VCE that were subsequently detected on other imaging modalities such as DBE, CTE or MRE. These lesions were mostly in the proximal small bowel which can be poorly visualized on VCE. This has been evidenced by an earlier study demonstrating that the ampulla of Vater being missed in > 50% of capsule examinations[33]. In a study by Baichi *et al*[34], VCE was performed on 300 consecutive patients presenting with OGIB. Among those patients, 10 small bowel masses were identified, and of those lesions noted, three duodenal masses were missed on previous EGD, with one missed on VCE as well. Further evidence of VCE missing lesions in the proximal small intestine came from a study by Postgate *et al*[35] who reported five tumors missed on VCE for evaluation of OGIB. Three of these tumors were in the distal duodenum, one in the proximal jejunum, and the fifth was a large Peutz-Jegher's polyp in the proximal ileum.

In a study by Selby *et al*[36], capsule endoscopes with varying field of view were evaluated for the ability to identify the ampulla of Vater. The ampulla of Vater was seen in 18% of PillCam SB2 that has a wider field of view (156°) compared to 0% of PillCam (140°). The PillCam SB3 has a variable frame rate of 6 fps when moving fast. It remains to be seen how effective this enhancement will be in visualizing the ampulla of Vater and allowing for better identification of proximal small bowel lesions in the future.

Timing of VCE

The diagnostic yield of VCE for the evaluation of OGIB has been demonstrated to be higher if VCE is performed soon after the onset of bleeding. Of one hundred consecutive patients evaluated for obscure GI bleeding, Pennazio *et al*[9] reported 92.3% positive yield in patients with ongoing overt bleeding (n = 26), 12.9% yield in patients with previous overt bleeding (n = 31) and 44.2% in patients with guaiac positive stools and iron deficiency anemia (n = 43). Similarly, Bresci *et al*[37] reported a positive yield of 91% in patients who underwent VCE within 15 d of obscure GI bleed event versus a yield of 34% in patients who underwent VCE placement after 15 d. Goenka *et al*[38] reported that out of 385 patients investigated for obscure GI bleed, patients with VCE placement within 48 h of overt GI bleed had the highest diagnostic yield (87%). This was significantly greater ($P < 0.05$) compared to patients who had VCE placed after 48 h (68%) of overt GI bleed, as well as those with occult obscure GI bleed (59%).

Recently our group reported that early use of VCE (*i.e.,* within 3 d of hospital admission) led to improved diagnostic yield, higher rate of therapeutic interventions, along with decreased hospital length of stay[39]. In the early deployment group, VCE findings of active bleeding or vascular angioectasia were significantly higher than in the group where VCE was deployed late (after more than 3 d of admission), or in the group where VCE was performed as an outpatient (44.4% *vs* 27.8% *vs* 25.8%). Therapeutic intervention was also carried out more often in the early deployment group compared to the other groups (18.9% *vs* 7.4% for the late group *vs* 10.3% for the outpatient group). Hospital length of stay was also shorter at 6.1 d in the early deployment group, compared with 10.3 d in the late deployment group.

Second look video capsule endoscopy

Several studies have shown that VCE can detect a bleeding site in 45%-66% patients with OGIB, and it is often felt that patients with OGIB and a negative VCE had a low rate of re-bleeding. However, in a study looking at the outcome of 35 patients who had negative VCE for

the evaluation of OGIB, the overall re-bleeding rate was 23% (8 patients) at a median of 15.9 mo of follow up. Four of these patients underwent repeat endoscopy after negative VCE and found previously missed lesions with potential as a bleeding source in the stomach. Overall 13 patients (37%) with or without re-bleeding underwent repeat endoscopy after a negative VCE, which lead to a definitive diagnosis in nine patients (69% who underwent repeat endoscopy). Lesions were located in the stomach and colon in eight of these nine patients[40]. In another study, Vlachogiannakos *et al*[41] noted that in 317 VCE performed for the evaluation of OGIB with negative prior upper endoscopy and colonoscopy, a bleeding source was found on VCE to be outside the small bowel in 3.5% cases, typically duodenum or cecum that was missed by conventional upper endoscopy or colonoscopy. Min *et al*[42] found a higher diagnostic yield for back-to-back VCE and showed that for a single VCE, yield was 37.5%, which increased to 43.8% with a second VCE, and up to 62.5% with back-to-back VCE. Therefore repeat VCE and/or endoscopic evaluations are recommended in cases of severe anemia, or persistent obscure/overt GIB. Timing of VCE is also important as discussed above.

TECHNOLOGICAL ENHANCEMENTS IN CAPSULE ENDOSCOPY

Technological enhancements

Abnormal findings may only be present in a few image frames, and the usefulness of VCE relies on accurate detection of these fleeting images. Several features have been built-in to capsule endoscopy software with the goal of improving detection, such as Suspected Blood Indicator (SBI) and flexible spectral imaging color enhancement (FICE). Different software programs are also equipped with special viewing modes to decrease reading time, such as with QuickView (Given Imaging) and "auto-speed-adjusted" and "express-selected" playback modes (Olympus).

Suspected blood indicator: SBI automatically highlights frames containing several red pixels in an attempt to help capsule reader localize bleeding source. However, use of the SBI is identifying clinically significant lesions is limited by its low sensitivity 56.4% and specificity 33.5%. SBI only has a 24.0% positive predictive value and 67.3% negative predictive value[43]. Intra-luminal bubbles created many of the false positive results. Anecdotally, SBI does have value in overt bleeding where it becomes a solid red bar, the proximal end of which nearly always marks the site of bleeding.

Flexible spectral imaging color enhancement: FICE is an image enhancement system that can obtain bright and high-contrast images. In a study looking at the ability of FICE to detect angioectasia as compared with conventional images, the sensitivity and specificity of detecting angioectasia with FICE images were 91% and 86%, compared with 80% and 100% with conventional mode[44]. FICE reading resulted in more false positive lesions, which can be correctly identified by converting the images to conventional mode.

Quickview system: The QuickView system scans each frame and analyzes patterns/colors to select significant images to create a short video that can then be a quick preview of the entire capsule. Even though QuickView mode may reduce reading time, prospective trials showed a high 8%-12% miss rate[45-47], and it is not recommended as a substitute to reading the entire capsule study.

Auto-speed-adjusted and express-selected playback modes: Olympus capsule endoscopy software systems have equipped an "auto-speed-adjusted" and "express-selected"playback modes. There is also an overview feature which is a one page summary of selected still images which provides the reader with a quick glance of characteristic frames from the capsule study. In the "auto-speed-adjusted" mode, the software speeds up the fps of the video to a maximum of 25 fps when the software detects repeated images similar to the previous frames, thereby potentially reducing the reading time. In the "express-selected" viewing mode, the software skips similar images, and produces a running video stream of only dissimilar images for viewing by the reader. Those skipped images are then gathered into the "expressed-skipped" mode for subsequent viewing if necessary. In a retrospective study of 70 patients to evaluate the clinical efficacy of these functions, Subramanian *et al*[48] noted that the capsule reading time using "express-selected" mode with the overview feature was much lower (19 ± 5 min) than using "auto-speed-adjusted" mode with the overview feature (34 ± 10 min). The missed rate was 8% when the overview function was used alone, but decreased to 0.03% when the overview function was combined with either "express-selected" or "auto-speed-adjusted" playback functions. Though this appears to be promising, further prospective evaluation in a large multicenter trial is needed before this could be recommended for widespread use in clinical practice.

Localization

The clinical problem: While video capsule endoscopy (VCE) is the gold standard for diagnosis of small bowel bleeding, endoscopists are still faced with the clinical challenge of localizing bleeding sources identified by VCE[49,50]. There are many issues at play here. First, the small intestine is a featureless tube which offers only two reliable landmarks for endoscopists - the pylorus and the cecum. Second, the small bowel is stacked upon itself in the peritoneal cavity, meaning that the capsule will traverse through multiple planes as it relates to a single point on the abdominal wall.

Because capsule transit time from the pylorus to the cecum is consistently about four hours, current clinical

practice involves identifying time points associated with the pylorus and cecum and then noting the time point associated with visualization of the bleeding source. Endoscopists can then approximate the distance of the lesion between these two landmarks. One issue with this technique is that if the capsule is not able to visualize the cecum (*e.g.,* because of slow transit time), localization solely based on knowing the time point associated with the pylorus becomes very inaccurate[51,52].

Since the advent of the video capsule, multiple studies have been undertaken to provide a more definitive system of localization. The two major techniques being studied are magnetic field and radiofrequency (RF) localization.

Magnetic field localization: Unlike radiofrequencies, magnetic fields are unaffected by human tissue, allowing for more accurate localization. Magnetic field-based systems also allow the opportunity to control the movement of the capsule while it travels the small bowel using one system[53,54]. Using one system for both capsule control and localization, however, will produce interference that can obstruct both of these functions. Given the accuracy required to control the movements of a capsule through the bowel, this system may not be appropriate. A second issue with magnet-based localization is its application in the clinical setting. Specifically, an examination performed using this system would require a space containing no ferromagnetic materials. Also, magnet-based systems are very complex and would be difficult to utilize in clinics[55].

Radiofrequency localization: An issue surrounding RF-based systems is that radiofrequencies do not easily travel through human tissue. In an effort to better understand the behavior of radiofrequency signals within the human body, a series of multidisciplinary conferences have been convened to address the problems associated with what is called body area networking[56,57]. What is clear is that RF within the body is influenced by multiple factors including tissue densities, juxtaposition of different organs, and other anatomic considerations. Despite this, one advantage of RF-based systems is the ease of utilization in the clinical setting. The first commercially available localization system was the RF-based system attached to the M2A capsule developed by Given Imaging[58]. This localization system has been discontinued due to its inaccuracy. In addition, this system only produces localization data in two dimensions and, therefore, its clinical utility is decreased since capsules travel in multiple planes. Marya *et al*[59] recently reported on a new RF-based localization system developed by Olympus Medical Systems for the new EC-10 capsule. This system has similar accuracy to the Given system while providing three-dimensional localization instead of only two dimensions. The clinical utility of this system cannot be properly assessed until a prospective trial is performed.

Future considerations: Although progress is being made in the development of new localization systems, there are still issues to be addressed. Research needs to be focused on developing a localization system that provides information related to the distance the capsule travels from the pylorus to the suspected bleeding lesion. It is this distance, not simply the three-dimensional location of a capsule within the abdominal cavity, which has the greatest clinical utility to an endoscopist or surgeon in the management of obscure GI bleeding.

MANAGEMENT OF OGIB

Therapeutic options

While progress is being made in the diagnosis of lesions contributing to obscure GI bleeding, the clinical challenge of treating the suspected lesions persists. Traditionally, analysis of particular therapeutic options has been limited to case series or small clinical trials. The decision to choose a particular option is based on several factors. Specifically, clinicians consider where the suspected lesions are within the GI tract, the number of suspected lesions at risk of further bleeding, the degree of bleeding and anemia, co-morbidities and the severity of symptoms experienced by the patient before deciding on a particular therapeutic intervention.

Endoscopic therapy

Once an obscure lesion is localized through endoscopy, the endoscopist has several options available for treatment. Treatments include APC and endoscopic band ligation (EBL).

Argon plasma coagulation: APC therapy is the gold standard therapy for gastric antral vascular ectasias (GAVE) and is widely used to treat angioectasia throughout the GI tract[60-62]. A prospective study by Kwan *et al*[63] provided definitive evidence of the usefulness of APC therapy in 100 patients with both angioectasia and GAVE. In their study the authors found that in a previously transfusion-dependent subset of subjects, over half did not require further transfusions post-APC. In a smaller study population, Herrera *et al*[64] demonstrated a 90% success rate for APC therapy in patients with focal vascular ectasias. In that study, APC was not associated with any adverse effects. Other studies have reported a 2.5% rate of adverse events[65].

Endoscopic band ligation: Historically, EBL has been a treatment for esophageal varices, but its usefulness as a treatment for GAVE and other angioectasia throughout the GI tract is now being realized[66]. In a study of 22 patients, Wells *et al*[67] demonstrated the benefits of EBL, as a subgroup receiving the therapy required fewer treatment sessions and had better-controlled bleeding than those receiving thermal therapy. Earlier studies have shown an equal efficacy and safety profile for EBL in the treatment of Dieulafoy's lesions compared to hemostatic clips or injection therapy[68].

Future directions: Despite important advances in treatments, a large prospective study comparing endoscopic therapies is lacking. As diagnostic measures improve, such a study could prove vital in allowing endoscopists more opportunities to treat obscure GI bleeds.

Pharmacologic therapy

If multiple lesions are suspected to be throughout the GI tract, or if a patient is found to have persistent bleeding despite repeated endoscopic or surgical interventions, pharmacologic therapy should be considered. Pharmacologic therapy may also be pursued for patients with multiple medical co-morbidities that may make them poor candidates for repeated endoscopy or surgical interventions.

Hormonal therapy: The utilization of hormonal therapy (*i.e.,* estrogen plus progesterone) for the treatment of suspected vascular malformations in the GI tract originated from the treatment of hereditary hemorrhagic telangiectasia (HHT, also known as Rendu-Osler-Weber disease). Much of the support for this therapy, however, came only from case reports or from studies with very small sample sizes.

van Cutsem *et al*[69] demonstrated a significant benefit of hormonal therapy compared to placebo (failure rate of therapy 29% compared to 100% for placebo). Despite impressive results, there are many significant issues with this study. Specifically, the sample size of patients was small and the study population included several individuals with HHT who represent a small sub-population of patients with OGIB[69]. These results have been countered by other studies including a larger more recent study from Junquera *et al*[70], which demonstrated no definitive benefit from hormonal therapy compared to the placebo. As other studies suggest, the pathogenesis of vascular malformations in the GI tract is quite different from the process associated with HHT[71,72]. Currently, there is no definitive evidence for the efficacy of hormonal therapy in GI bleeding that is unrelated to HHT.

Anti-angiogenics: The pathogenesis of vascular malformations related to GI bleeding provides multiple options for therapy. In particular, much focus has been placed on the role of vascular endothelial growth factors (VEGF) in the development of these lesions. Junquera *et al*[73] demonstrated that patients with recurrent bleeding secondary to intestinal angiodysplasias (AD) have accumulations of VEGF along the endothelial lining of colonic resection specimens. Research suggests that VEGF becomes overexpressed in oxygen-depleted mucosa, contributing to the formation of AD in older tissues[74,75]. This role of VEGF in the development of AD has created a niche for anti-angiogenic therapy in AD-associated bleeding.

Thalidomide (which has been used in Crohn's disease patients due to its anti-TNF effects) is now being studied in patients with AD-related bleeding. Recently, Ge *et al*[76] performed a randomized controlled trial demonstrating the efficacy of thalidomide in treating AD, 71.4% of patients responded, compared to 3.7% in the control group. Although these results are promising there are significant adverse events associated with thalidomide therapy including leukopenia, deep vein thrombosis, and peripheral neuropathy[77]. Bevacizumab is another anti-angiogenic medication recently studied as a VEGF-inhibitor. Studies have demonstrated its usefulness as an antiangiogenic medication for colon and renal cancer, but there have been no formal studies performed in patients with recurrent GI bleeding from suspected vascular malformations[78].

Somatostatin analogues: The most well-studied somatostatin analogue for the treatment of OGIB is octreotide. The suspected mechanism of action is the ability to inhibit the production of intestinal enzymes (*e.g.,* cholecystokinin, gastrin, and vasointestinal peptide), decrease splanchnic blood flow, decrease platelet aggregation, and decrease angiogenesis. One case series by Nardone *et al*[79], demonstrated that octreotide treatment stopped bleeding in 10 of 17 patients. The authors noted that patients receiving octreotide therapy experienced few side effects. The most common side effects of octreotide therapy are abdominal discomfort and diarrhea, considered to be relatively mild compared to side effects of some of the other therapies listed here[80].

Future directions: While the pathogenesis of AD-related bleeding offers multiple promising opportunities for intervention, a focus on developing randomized-controlled trials to better assess these therapies is needed to define their clinical utility and potential side effects.

Surgical therapy

With advances in endoscopic techniques that allow for visualization and treatment of lesions responsible for OGIB, surgery has become less of a necessity. Now, surgery may be pursued in patients who have failed medical and endoscopic therapy, as well as patients who present with an acute hemorrhage. But in all cases a target lesion needs to be defined preoperatively to avoid the high likelihood of a negative exploration. Research in this field has focused on the localization of lesions to allow surgeons the opportunity to make a curative resection. Studies have demonstrated the benefits of injecting methylene blue dye as an intraoperative technique to allow surgeons to identify the areas of the bowel affected by AD[81,82]. Although this technique has been used since 1978, recent studies have suggested adaptations to make the process easier. For example, D'Mello *et al*[83] presented a case report using digital subtraction angiography to reveal a vascular malformation which they then accessed easily using a microcatheter. Further investigation into capsule endoscopy localization and intraoperative localization of lesions will allow surgeons to make more definitive resections while decreasing the length of bowel needed to be removed.

RECURRENCE OF OGIB

One of the biggest challenges associated with OGIB is that of recurrence. The rate of re-bleeding varies in literature depending on center, duration of follow up and cause of bleeding. Studies have demonstrated the re-bleeding rate to be in the range of 40%-60% when associated with a finding of angiodysplasia on VCE[84,85]. Endo *et al*[86] studied the rate of re-bleeding after intervention for lesions detected on VCE, and found 50% re-bleeding rate in patients with angiodysplasia despite endoscopic intervention. The re-bleeding rate was also higher for patients with clinically insignificant lesions, regardless of whether endoscopic intervention was performed[86]. Patients requiring multiple transfusions for recurrent bleeding typically have multiple co-morbidities, such as chronic renal failure or use of anticoagulation, that are also independent risks for re-bleeding. Patients with recurrent bleeding require multiple endoscopic procedures and are thus at increased risk of complications from these procedures.

Another challenge associated with recurrent OGIB is negative endoscopic findings on VCE or deep enteroscopy. Evidence is conflicting in this area with some studies showing a higher rate of bleeding with normal mucosa or insignificant lesions on endoscopy[84], whereas others show higher rate of recurrent bleeding associated with positive findings on VCE[85]. Studies have reported re-bleeding rate as low as 5.6% and 11% in patients with negative VCE[85,87]. Koh *et al*[88] investigated long-term outcome in OGIB after negative VCE, and found that the overall re-bleeding rate was 28.4%. The re-bleeding rate was higher in patients with positive VCE (36.8%) than in those with negative findings (22.8%)[88]. It is also reported that in VCE-directed interventions, 50%-66% of patients remain transfusion-free without recurrent bleeding[89,90].

FUTURE IN THE DIAGNOSIS AND MANAGEMENT OF OGIB

Advances in capsule endoscopy have included longer battery life, higher image capture frame rate, wider angle of view, improved image resolution, along with enhanced software features to assist in reading. Studies suggest that there is room for further education in reading VCE videos. The more widespread use of early capsule deployment in overt OGIB should enhance diagnostic yields and increase therapeutic intervention rates. The ability to define distance travelled by the capsule would be very helpful in lesion localization. The new 3-D localization software is a step in the right direction. Difficult to detect sources of bleeding may be better controlled by medical means and should stimulate drug development and clinical trials of such agents. The tools for deep enteroscopy are likely to evolve shortly. However, cost constraints for these procedures would preclude their primary deployment in most parts of the world. Thus VCE and DE are likely to remain complimentary procedures for the foreseeable future.

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REVIEW

Pathophysiological mechanisms linking obesity and esophageal adenocarcinoma

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Abstract

In recent decades there has been a dramatic rise in the incidence of esophageal adenocarcinoma (EAC) in the developed world. Over approximately the same period there has also been an increase in the prevalence of obesity. Obesity, especially visceral obesity, is an important independent risk factor for the development of gastro-esophageal reflux disease, Barrett's esophagus and EAC. Although the simplest explanation is that this mediated by the mechanical effects of abdominal obesity promoting gastro-esophageal reflux, the epidemiological data suggest that the EAC-promoting effects are independent of reflux. Several, not mutually exclusive, mechanisms have been implicated, which may have different effects at various points along the reflux-Barrett's-cancer pathway. These mechanisms include a reduction in the prevalence of *Helicobacter pylori* infection enhancing gastric acidity and possibly appetite by increasing gastric ghrelin secretion, induction of both low-grade systemic inflammation by factors secreted by adipose tissue and the metabolic syndrome with insulin-resistance. Obesity is associated with enhanced secretion of leptin and decreased secretion of adiponectin from adipose tissue and both increased leptin and decreased adiponectin have been shown to be independent risk factors for progression to EAC. Leptin and adiponectin have a set of mutually antagonistic actions on Barrett's cells which appear to influence the progression of malignant behaviour. At present no drugs are of proven benefit to prevent obesity associated EAC. Roux-en-Y reconstruction is the preferred bariatric surgical option for weight loss in patients with reflux. Statins and aspirin may have chemopreventative effects and are indicated for their circulatory benefits.

Key words: Adipose; Body mass index; Reflux; Barrett's esophagus

Core tip: Excess adipose tissue, particularly visceral obesity, is an important risk factor for esophageal adenocarcinoma (EAC). The mechanisms involve both the promotion of gastro-esophageal reflux and reflux-independent mechanisms. Abnormal secretion of the adipokines leptin and adiponectin from adipose tissue in obesity may promote the development of EAC. Increased leptin levels are an independent risk factor for EAC and leptin enhances proliferation and invasion and inhibits apoptosis in Barrett's cell lines. Relative adiponectin deficiency is an independent risk factor for EAC and adiponectin blocks the cancer promoting effects of leptin in experimental models. Obesity may influence EAC development *via* adipokine secretion.

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INTRODUCTION

Esophageal adenocarcinoma is a health problem of increasing global health significance. The overall prognosis of esophageal adenocarcinoma (EAC), the most prevalent form of esophageal cancer in the developed world, is dismal, with a 5-year survival of 15%-20% at best[1]. At the same time the incidence of this cancer has increased dramatically, by approximately 600% in the last 30 years, leading some commentators to call this an epidemic[2]. A detailed understanding of the pathogenic mechanisms leading to this malignancy is required to enable the development of strategies for both prevention and treatment. Over a similar period the prevalence of obesity has increased in the developed world and this increase in obesity has been linked with increased risks of several cancers, including oesophageal adenocarcinoma (OAC)[3,4]. Over the last 30 years rates of obesity have been increasing steadily, most obviously in the United States and Western Europe[5] but also in lower and middle income countries[6]. Estimates from The World Health Organisation suggest that 12% of the world's population aged over 20 years old is now obese which equates to approximately 500 million adults. It is estimated that 10% of men and 14% of women are obese by standard criteria. This has doubled from the 1980s[6].

Well-designed epidemiological investigations have been instrumental in detecting and defining the association between obesity and EAC. Relevant features of the association have been explored in detail to determine features of causality and to help clarify the potential implicated mechanisms through which obesity may act. This review summarises relevant recent observational data on the association between measures of obesity and risk of EAC, gastro-esophageal reflux and Barrett's esophagus; and experimental data of plausible mechanisms contributing to carcinogenesis and explores where and how interventions may reduce the burden of this disease.

CLINICAL MEASURES OF ADIPOSITY AND METHODOLOGICAL CONSIDERATIONS IN RELEVANT OBSERVATIONAL RESEARCH

The World Health Organization definition for overweight and obesity is abnormal or excessive fat accumulation that may impair health. Adiposity has been quantified in observational research relevant to this topic using a number of measures including anthropometric measurements and imaging. Overall adiposity is commonly measured using body mass index (BMI) [weight (kg) per height squared (m^2)], and central adiposity (synonymous to visceral and abdominal adiposity) is measured using waist-to-hip ratio (WTHR), waist circumference and anterior-posterior abdominal diameter (cm); and imaging such as visceral adipose tissue area (m^2) (VATA) or volume (m^3) as determined by computerised tomography or magnetic resonance imaging. While BMI is a simple measure of overall adiposity that is practical for large epidemiological studies, it is crude and does not necessarily reflect the varying proportions of fat and lean (non-fat) mass or body fat distribution. Measures of central obesity have been demonstrated to vary substantially within a narrow range of BMI, whilst central obesity itself is a combination of subcutaneous obesity around the abdomen and mesenteric adipose tissue. Furthermore, as body composition changes with age, and height decreases, for example through kyphosis and loss of vertebral height, BMI may be overestimated in older participants[7]. Imaging modalities used to quantify fat distribution, can precisely estimate the size of body fat compartments. However, they are less suitable for large scale prospective studies: they are often performed at diagnosis rather than for preceding time points and could therefore underestimate associations depending on weight loss associated with the diagnosis; and their use requires convenience sampling for controls (*e.g.*, patients undergoing investigation for other reasons), and therefore may be less representative of the population under study. These are important considerations when appraising the evidence on the risk of reflux, Barrett's esophagus and EAC with adiposity.

OBESITY AND RISK OF ESOPHAGEAL ADENOCARCINOMA

The association between measures of obesity and risk of OAC has been extensively examined in epidemiological investigations. The most striking evidence for a potential causal association between adiposity and risk of this cancer is the wealth of consistent data to suggest the association is among the strongest than for any other malignancy with evidence of a biological gradient[4]. A systematic review of prospective studies from Europe, Australia and the Asia-Pacific region, that measured BMI at baseline and followed participants until the development of incident cancer (hence supporting a temporal relationship), included 1315 male cases of OAC and 735 female cases, demonstrated the magnitude of the association in men was stronger than for any other malignancy, from 16 sites; and in women was only second to endometrial from 19 sites. The strength of the associations (per increase in BMI by 5 kg/m^2) was almost the same in both genders (RR = 1.52, 95%CI: 1.33-1.74 for men; RR = 1.51, 95%CI: 1.31-1.74 for women) with minimal heterogeneity. This implies the association between BMI and risk of EAC is consistent between well-designed prospective studies, further supporting the causality, and that sex-specific differences in the incidence of EAC are likely unrelated to adiposity as measured by BMI. Interestingly,

for squamous cell carcinoma, the most common histological type of esophageal cancer worldwide, in both men and women, risk was significantly reduced with increased BMI, more strongly than for any of the included malignancies[4]. A recent meta-analysis of five observational studies, including four prospective studies[8-11] and one case-control study[12], reported a significant association between abdominal obesity (as a composite measure of VATA, WHR, AC and abdominal diameter) and risk of EAC (adjusted OR = 2.51; 95%CI: 1.56-4.04)[13]. Indeed, the effect of abdominal obesity, as measured by WHR or AD, on risk of OAC has been reported to be independent of BMI[8,10]. This implies a role of abdominal obesity in the pathogenesis EAC over and above general obesity. Furthermore, the association between general or central adiposity and risk of EAC has been demonstrated to persist despite inclusion of plausible confounders in multivariable analyses, including: symptomatic reflux, physical activity, smoking, and intakes of total energy, red meat, fruit and vegetables[10].

Although the available cohort studies have not clearly shown that visceral adiposity is associated with an increased risk of invasive neoplasia in patients with Barrett' s esophagus, two recent studies have suggested that increased visceral fat tissue[14] or total abdominal obesity[15] are associated with the progression to high-grade dysplasia. Equally a recent meta-analysis including measures of central adiposity at least 5 years before the diagnosis of EAC showed a significant increased risk of cancer with central obesity[13]. Given our understanding of the biology of Barrett's esophagus, these data do suggest that central obesity promotes cancers in patients with Barrett's esophagus.

OBESITY AND RISK OF GASTRO-ESOPHAGEAL REFLUX

A commonly proposed mechanical explanation for the associations between obesity and EAC is through the following sequence: increased abdominal adiposity leading to increased intra-abdominal pressure, then consequent reflux predisposing to Barrett's esophagus and then EAC[16]. While either abdominal or central adiposity has been associated with each of these "steps"[7,17-19] it has not been possible so far to empirically demonstrate this whole sequence to be causal[17]. Measures of central obesity appear strongly associated with symptomatic reflux, independent of BMI, in a dose-dependent manner[20]. However, in patients with reflux, for each kg/m^2 increase in BMI, while both intra-gastric pressure and gastro-esophageal pressure gradient (GEPG) rise[18]; increments in GEPG are not associated with acid exposure as determined by 24-h pH monitoring. Therefore, obesity does not appear to promote reflux through a purely mechanical means, which suggests alternative obesity-induced mechanisms of esophageal dysfunction are operating. While increased reflux could feasibly contribute to the increased risk of EAC observed in obese persons, other mechanisms are likely at play as obesity is strongly associated with risk of EAC, independent of symptomatic reflux. A recent well-conceived Swedish population-based case-control study, which included 189 incident cases of EAC and 816 population-based controls, demonstrated the effect sizes for overweight BMI categories (> 25 kg/m^2) verses underweight BMI category (< 25 kg/m^2) on risk of EAC were not significantly different in adjusted or unadjusted models for severity, frequency or duration of reflux[21]. This study demonstrated significant synergy between BMI and reflux, most strikingly for frequency (for more than 3 times per week), but also for severity and duration of reflux, on risk of EAC. Other epidemiological studies have also demonstrated the reflux-independent effects of BMI[8,22-24] and abdominal obesity[8] on the risk of EAC, however, It should be noted these studies rely on the reporting of symptomatic reflux and do not necessarily reflect the actual amount of acid reflux.

It might be the location of the fat rather than pure BMI that is important. Abdominal obesity, rather than excess weight has been suggested as the true association of the increase in GORD. The association between BMI and GORD was attenuated when adjusted for waist circumference suggesting BMI has its affect by increasing abdominal obesity[25].

Although one large cross-sectional study found no association between GERD and waist circumference or waist: hip ratio[26], a considerable body of research suggests that an enlarged waist circumference increases the risk of erosive oesophagitis[27-29]. A Korean study of 5329 subjects reported an association between abdominal visceral adipose tissue volume, but not BMI or waist circumference, and erosive esophagitis[30]. Visceral adipose tissue has been assessed by CT scan and high levels of visceral adipose tissue were significantly associated with the duration of GERD symptoms[31]. The association is most obvious in the white population, which could help explain the high levels in the developed world. It is not associated with black or Asian ethnicities[20].

ADIPOSITY AND RISK OF BARRETT'S ESOPHAGUS

In a recent pooled analysis from the BEACON consortium, including 1102 cases of long segment BE (> 3 cm) and 1400 population-based controls from four case-control studies, increasing waist circumference was significantly associated with risk of BE, independent of BMI (OR = 1.87, 95%CI: 1.22-1.32) for the highest *vs* lowest quartile), with evidence of a significant biological gradient (OR = 1.16, 95%CI: 1.02-1.32, per 5 cm increase in waist circumference)[19]. The effect sizes for the association between waist circumference and risk of BE were similar in both men and women and were almost unchanged after adjustment for symptomatic reflux.

A meta-analysis reported that BMI per se was not associated with BE[32] but that increased waist circumference that confers a two-fold risk for BE[33]. Further

studies have reported similar findings as to the effect of visceral obesity on the risk of BE but have also shown an inverse relationship with glutofemoral obesity[34,35]. This could be due to the less metabolically active nature of glutofemoral adipose tissue which further supports the theory it is distribution of adipose tissue, not just overall increase in weight or the excess fat tissue that is a risk for BE. Recent studies have shown that even abdominal obesity is too crude a tool: it appears to be the visceral (mesenteric) component rather than the subcutaneous component that is the most important risk factor for BE[31,36].

Furthermore, the preponderance of BE in men is not explained by differential risk in men and women according to BMI alone. Although waist circumference increased the risk of BE in male and females, the association in females but not males is attenuated when adjusted for GORD symptoms[37]. It is possible that non-reflux related mechanisms contribute more to development of BE in males and these extra mechanisms could explain the higher male prevalence of BE. Abdominal subcutaneous fat was not associated with the development of BE, whereas visceral adiposity was[14].

The pathogeneses of GERD, BE and cancer are complex and multifactorial[38]. It is important to note that symptoms of GERD are fairly uniformly distributed globally (albeit generally less prevalent in Eastern countries compared to Western or Middle Eastern[38]) but the burden of erosive esophagitis, Barrett's esophagus and adenocarcinoma becomes increasingly concentrated in white males in the Western world[38]. Whilst this has an important correlation with exactly the group with the greatest increase in visceral obesity, it does limit the global generalizability of the data; whilst the links between obesity and GERD are generally consistent worldwide, the majority of the epidemiological data related to obesity, Barrett's and cancer, are from this most prevalent group and it is possible, although unproven that other factors may be more important in other racial groups or geographical areas.

WEIGHT LOSS AND RISK OF EAC

Unsurprisingly, to date there are is no significant body of literature on the effect of interventions to promote weight loss as a means to reduce the risk of BE or EAC. At a general population level the age-standardized incidence of EAC is relatively low (approximately 12 per 100000 per year in the United Kingdom)[2] and therefore a randomised controlled trial would require an unfeasibly large sample size to empirically demonstrate this. A clinical trial to determine whether or not a weight loss programme could reduce the risk of EAC in a group at higher risk of progression, such as those with known BE, may be more feasible. However, such a clinical trial would be problematic in interpretation as causality could not be attributed to obesity (or lack of) *per se*, but ascribed to the intervention designed to promote weight loss, which may plausibly act through a number of pathways (*e.g.*, exercise and diet). There is evidence that weight loss secondary to lifestyle, dietary changes or surgery is associated with a reduction in symptomatic reflux[39]. Ascribing causality to obesity on the risk of EAC can therefore only be determined through comprehension of the available epidemiological data on the key features of the association, which are consistent with a causal relationship, and an appraisal of laboratory data.

MOLECULAR MECHANISMS

Whilst the pathogenesis of EAC is not fully defined, increasingly the molecular changes are being understood[40]. Detailed discussion of the cellular and molecular changes leading to the development and persistence of the clone(s) of cells which give rise to initially Barrett's Esophagus and the later progression in some cases to adenocarcinoma are outside the scope of this review[41,42] but it seems clear that reflux of gastro-duodenal contents is involved in the initiation, perpetuation and progression of the esophageal changes. However there must be other factors also driving these changes. As reviewed above, there are considerable epidemiological data linking various markers of obesity with the development of EAC and several, not mutually exclusive, biologically plausible mechanisms will be explored (Figure 1). However exactly how these mechanisms associated with obesity interact to promote EAC remains unclear, but exploration of these mechanisms is likely to be fruitful in order to explore new treatment and preventative therapies.

POSSIBLE MECHANISMS LINKING OBESITY WITH ESOPHAGEAL ADENOCARCINOMA

The association is by chance

It is first necessary to consider that the association is merely chance and that obesity does not directly contribute to the pathogenesis of EAC. There have generally been parallel increases in obesity and EAC in the last few decades, and as discussed previously, obesity (especially abdominal visceral obesity) is clearly a risk factor for Barrett's esophagus and EAC[43]. Some inconsistencies in the data deserve further comment: there have been dramatic rises in the incidence of EAC incidence Australia and Denmark but with much more modest changes in obesity. The epidemic of EAC in the United Kingdom appeared to start about 10 years before that in the United States, yet the United Kingdom was about 10 years behind the United States in the increase in obesity rates[2]. Despite these uncertainties, the vast majority of the epidemiology showing obesity as a risk factor of EAC is compelling and obesity is also associated with the risk of many other cancers. There is biological plausibility and the relative risk of EAC with obesity is higher than other cancers, all suggesting that the association is real even if

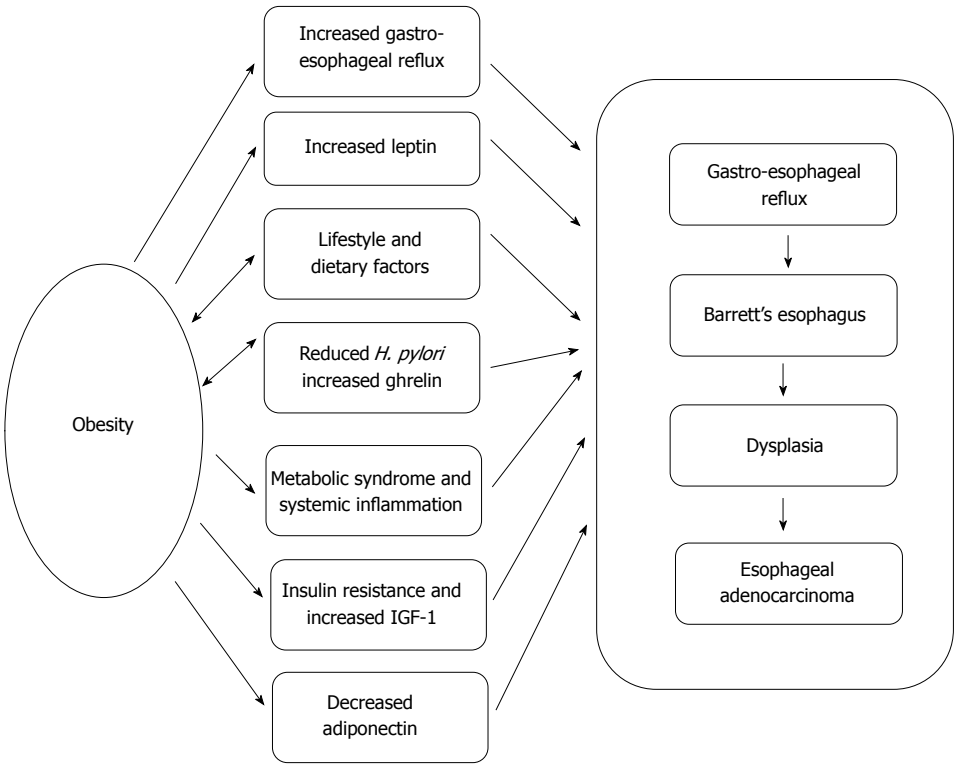

Figure 1 Possible mechanisms linking obesity with the development of esophageal adenocarcinoma. There are several potential and not mutually exclusive mechanisms that could link obesity and esophageal adenocarcinoma. Adipose tissue can exert both mechanical and endocrine effects that could enhance gastro-esophageal reflux and progression to adenocarcinoma. Decreased *H. pylori* could promote both gastro-esophageal reflux by increasing gastric acidity and increase body mass by enhancing production of the gastric appetite-stimulating peptide ghrelin.

obesity is not the sole driver of EAC[3].

Lifestyle or dietary factors associated with obesity increase the risk of EAC

It is possible that specific dietary or lifestyle factors associated with obesity promote EAC development. There are many potential individual variables in but no data specifically implicating any one factor. Smoking, for instance, is a risk factor for both Barrett's esophagus and progression to EAC[44,45] and lifestyle choices associated with significant obesity may be associated with greater proclivity to smoking but smoking is associated with a lower body mass, including patients with BE[46]. There is a complex and not completely understood inter-relationship between smoking, obesity, Barrett's esophagus and cancer. Whilst smoking does seem to be a consistent risk factor for progression to EAC[47], the effects on the development of BE are rather more variable; positive[46] and negative[48,49] associations have been reported and a meta-analysis concluded that being an "ever-smoker" was associated with an increased risk of BE when compared to population-based (OR = 1.42, 95%CI: 1.15-1.76) or non-GERD-controls (OR = 1.44, 95%CI: 1.20-1.74) but not GERD-controls (OR = (1.18, 95%CI: 0.75-1.86)[50]. In one study the positive association between EAC and smoking was removed after adjusting co-variables[51]. There are very limited data examining the combination of measures of obesity and smoking of the risks of BE and EAC. Hardikar *et al*[15] reported that the increased risk of progression to EAC associated with a high WTHR was only seen in male "never smokers" and not in male regular smokers. In a case-control study of endoscopy patients smoking was a risk factor for the development of BE: there was a suggestion that the risk associated with smoking was higher in the more obese (in those with BMI > 30, OR = 5.6, 95%CI: 1.7-18.3) than those of lower body weight (BMI < 30, OR = 3.0, 95%CI: 1.5-6.1)[46], but these were not statistically significant differences. Other studies have failed to show any interaction[23,51] or have not specifically explored any possible interaction[48,49,52]. The decline in the prevalence of smoking has occurred over the same period as EAC has increased and smoking would not explain the racial differences in EAC incidence. Thus although cigarette smoking itself seems to be a risk factor for BE and progression to EAC, there are insufficient data to implicate smoking as direct line between markers of obesity and development of EAC.

Although moderate-severe exercise acutely can precipitate gastro-esophageal reflux, regular exercise is associated with a lower rate of erosive oesophagitis and also protects against obesity[53]. It is possible certain dietary substances may promote both obesity and relaxation of the lower esophageal sphincter (LOS) so promoting reflux disease and EAC. Although there are few convincing data implicating any specific dietary constituents, several possibilities exist: it seems a high calorie content of meals

independently of fat content is most likely to provoke reflux[54] and chocolate promotes LOS-relaxation[53]. EAC is also associated with increased meat intake and reduced fruit and vegetable intake[55] and there are many other putative dietary components that could directly or indirectly promote EAC development in obese patients.

Increased gastro-esophageal reflux as the link between obesity and EAC

The link between EAC and fat tissue is much stronger for visceral obesity than overall obesity[43]. Perhaps the most obvious pathogenic link is that the visceral fat tissue exerts mechanical effects on the upper GI tract to promote gastro-esophageal reflux directly and hence the Barrett's-cancer sequence indirectly. There are considerable experimental data showing that acid and/or bile exert effects on the esophageal epithelium that would be expected to promote cancer (including stimulation of proliferation, inhibition of apoptosis, generation of free radicals[56-58], hence factors that provoke reflux would be expected to enhance the development and progression of Barrett's esophagus. There are some data to support this hypothesis: obesity is indeed associated with an increased prevalence and severity of reflux[59-61] and also with size of hiatus hernia[62], greater esophageal acid exposure[25], and increased transient lower esophageal relaxations[63]. However it seems likely that visceral fat tissue exerts both direct and indirect effects on the promotion of esophageal carcinogenesis, the majority of the data show that obesity is associated with Barrett's oesophagus and/or EAC independently of measures of reflux[21,31,64,65].

A separate factor or factors have increased EAC and obesity

One alternative hypothesis is that a separate factor or mechanism has promoted both obesity and EAC independently of each other. There are some data implicating *Helicobacter pylori* (*H. pylori*) infection in this situation. Infection of the stomach with *H. pylori*, particularly the CagA positive strains that provoke more intense gastric mucosal inflammation is inversely associated with both erosive esophagitis and BE[66]. The most plausible explanation for this is that infection and the resulting inflammation of the gastric body leads to a reduction in gastric acid secretion due to either local cytokine production[67] or more irreversible process due to the subsequent development of gastric atrophy[68]. Thus *H. pylori* infection would be associated with less reflux severe reflux disease due to relatively decreased gastric acid secretion. The prevalence of *H. pylori* infection has fallen, whilst the incidence of EAC has increased[69] over the last century. Weight gain is common after *H. pylori* eradication and hence a reduced prevalence of *H. pylori* could directly provoke more severe reflux disease and an overall increase in body mass. In a recent meta-analysis infection with CagA positive *H. pylori* was associated with a significantly lower risk of esophageal adenocarcinoma 0.74 (95%CI: 0.57-0.97), although no significant relationship was seen with CagA negative strains or between *H. pylori* and esophageal squamous cancer[70].

Changes in dyspeptic symptoms could underlie the weight gain but a more direct link between these two has been postulated *via* the role of gastric ghrelin. Ghrelin is a peptide hormone produced in, and secreted from, the P/D1 cells in the gastric body. Ghrelin stimulates appetite. *H. pylori* infection is associated with lower levels of gastric mucosa ghrelin and these mucosal levels increase after H. pylori eradication[71,72]. Hence it possible that lower levels of *H. pylori* infection are directly linked to obesity by increasing appetite. Whilst this is an attractive hypothesis and serum ghrelin levels have been shown increase after successful *H. pylori* eradication[73], this link between *H. pylori* status and circulating ghrelin has not be found consistently[74,75] to reliably increase after H. pylori eradication and higher plasma ghrelin levels have themselves been associated with both a lower incidence of erosive oesophagitis (possibly by enhancing gastric emptying[76] and also protection against esophageal adenocarcinoma[77]. However, more in keeping with this hypothesis high plasma ghrelin levels have been shown to be positively associated with the development of Barrett's esophagus[76]. The time course of *H. pylori* prevalence is not completely consistent with the changing epidemiology of EAC. The prevalence of H. pylori had been falling steadily throughout the 20th century well before the upsurge in EAC[69] and the increase in EAC incidence in Sweden seemed to begin in the early 1990s, well after the discovery and active treatment of H. pylori. The beginning of the upsurge in EAC in the United Kingdom began in the 1960s, well before the discovery of *H. pylori*. Hypothesizing that decreased gastric *H. pylori* infection as a direct cause of both obesity and EAC is also unable to explain the clear gender and racial differences in EAC[2] Therefore *H. pylori* infection and gastric ghrelin seem not be major contributors to the link between obesity and EAC but these potential mechanisms do outline the potential importance of factors influencing both appetite and mucosal biology.

Meta-inflammation and the metabolic syndrome

Adipose tissue is now recognised as a complex metabolically-active tissue, which secretes a variety of mediators that can have effects throughout the body. These mediators can conveniently, if rather simplistically be grouped into two: those relatively specific for adipose tissue, generally called adipokines or adipocytokines which include several important mediators including leptin, adiponectin, resistin and omentin, these are generally primarily involved in energy balance homeostasis and a second group of systemic cytokines that can be produced by a variety of tissues not limited to fat cells[3,78,79]. Most commentators now accept that obesity is a state of chronic low-grade, systemic inflammation, also termed "meta-inflammation". This is predominantly caused by the secretion of a variety of pro-inflammatory mediators by the fat tissue. These include tumour necrosis factor-α

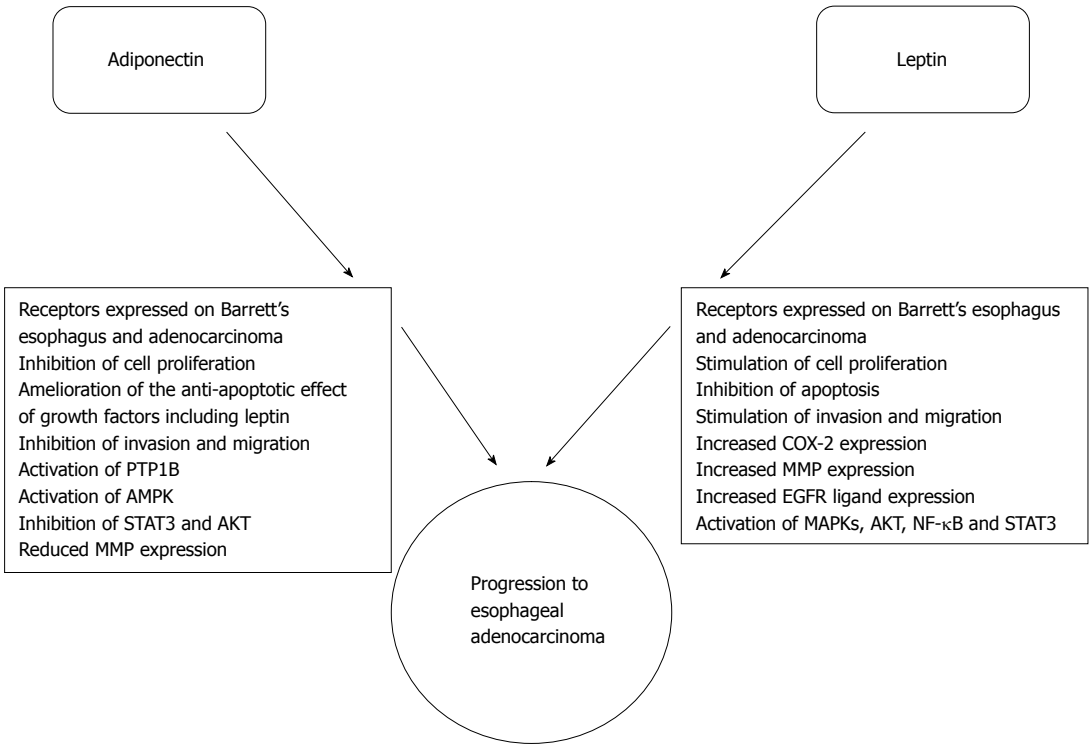

Figure 2 Effects of the adipokines leptin and adiponectin on Barrett's esophagus and esophageal adenocarcinoma. Obesity, more specifically visceral obesity, is associated with increased serum leptin and decreased serum adiponectin levels. Leptin and adiponectin have a set of antagonistic pathophysiological actions on Barrett's esophageal and adenocarcinoma cells.

(TNF-α), IL-1, IL-6, IL-8, interferon-β, MCP-1, VEGF and it is believed these mediators contribute not only to the development of the metabolic syndrome, with resulting insulin resistance and the related complications but also the increased risk of many cancers associated with obesity[79]. Systemic inflammation is recognised as a classical precursor to cancer, it is not completely understood how this systematic inflammatory state promotes cancer although, simplistically, many of these mediators promote cell proliferation, inhibit apoptosis and stimulate angiogenesis, all of which would be expected to promote cancer.

Faecal calprotectin, which is a marker of luminal inflammation, is increased in obesity[80]. There is an increased incidence of most gastrointestinal cancers associated with this obesity-induced inflammatory state[3], but the relative risk of EAC is higher than other cancers. Exactly how this meta-inflammation promotes EAC in the face of what would seem to be more severe and prolonged esophageal inflammation driven separately by acid and bile reflux is uncertain, although it again underlines the potential effects of circulating fat-derived mediators.

The meta-inflammation associated with obesity is associated with insulin resistance and increased circulating concentrations of both insulin and insulin growth factor-1 (IGF-1). This increase in insulin-related factors is at least partly driven by the secretions from metabolically-active visceral adipose tissue. As discussed above, a feature of obesity, and more specifically visceral obesity, is increased levels of inflammatory cytokine and mediators, including free fatty acids, TNF-α, leptin and resistin[81,82] and reduced secretion of adiponectin[83]. Insulin stimulates the production of IGF-1 and decreases production of the major serum proteins which bind insulin and IGF-1, insulin growth factor binding proteins 1 (IGFBP-1) and 3 (IGFBP-3)[84]. The overall effect is to increase the bioavailable levels of IGF-1. Both insulin and IGF-1 can bind to the insulin growth factor receptor complex, stimulating pathways that promote cellular proliferation. In a Barrett's cohort this insulin resistance has been associated with progression to adenocarcinoma[43]. Insulin and IGF-1 are mitogenic for many tissues, including Barrett's esophageal cells, which express IGF-1 receptors[85]. IGF-1 receptor expression is increased in EAC specimens resected from viscerally obese patients[86]. However the available data are conflicting on the role of IGF-1 and insulin as risk factors for malignant progression in BE. An increase in risk of cancer or BE have been reported[84,86], but other studies have failed to demonstrate any association between the metabolic syndrome and the risk of EAC [43] or between serum IGF-1 or IGFBP3 (the predominant serum binding protein) levels and progression of Barrett's[87].

Adipokines as effectors of the esophageal mucosal changes

These general inflammatory changes may be important in the development of EAC, but the specific role of adipokines is attracting considerable attention. Leptin and adiponectin have been examined in some detail and

it is possible they are a direct mechanistic link between obesity and progression to EAC (Figure 2). There are other adipokines such as resistin and omentin[88]: there no cell line or in vitro mechanistic studies examining the effects of these on esophageal tissues and there is a single epidemiological study showing that circulating resistin levels were higher in those with gastro-esophageal reflux disease than either Barrett's esophagus or controls[83] but further studies are required.

LEPTIN

Leptin is the archetypal adipokine. It is secreted as a 16 kDa protein from fat cells and serum levels are proportionate to body fat mass, as might be expected from it playing an important role as a regulator of appetite, energy metabolism and body weight. In the vast majority of obese subject, serum levels are significantly elevated and leptin deficiency is very rare cause of human obesity, in contrast to the gross obesity of the naturally occurring ob/ob leptin-deficient mouse[89]. It is thought that a degree of hypothalamic hyposensitivity to leptin is a more important cause of clinical obesity[90]. Many studies have reported that increased leptin levels are an independent risk factor for many cancers including breast, colorectal, prostate, ovarian, lung and endometrial[91]. Leptin levels have be an shown to be an independent risk factor for the development of Barrett's oesophagus[65,76,83], one study showed this effect was seen in male and not females[92] but another study confirmed this association in females[93]. Increased leptin levels have been shown to be an independent risk factor for progression to cancer in a cohort of patients with Barrett's esophagus[43]. Consistent with these data suggesting that leptin could directly affect the esophagus, leptin receptor expression has been detected in non-dysplastic Barrett's cell lines, esophageal adenocarcinoma cell lines, Barrett's esophagus and EAC[94-97]. One study has reported an association between increased leptin-receptor expression and more advanced stage in EAC[96].

Leptin promotes malignant behaviour in experimental esophageal cell line models. Leptin signals *via* the leptin receptor and increases proliferation, inhibits apoptosis, and stimulates migration and invasion. This is accompanied by the production of the matrix metalloproteinases (MMPs) MMP-2 and MMP-9 which are involved in invasion[94,98]. In a separate study, conditioned media from visceral adipocytes stimulated production of MMP-9 from esophageal adenocarcinoma cell lines and there is a clear association between *in vivo* MMP-9 production by EAC tissues and visceral obesity[99], suggesting that fat-derived mediators can influence esophageal epithelial behaviours, although the latter study did not confirm a specific role of leptin.

The cell signalling pathways involved in these leptin-induced effects have been well described[94,98]. Binding of leptin to the full-length receptor stimulates phosphorylation of the receptor-associated JAK2 tyrosine kinase which subsequently leads to activation of the protein kinase B/Akt and extra-cellular signal related kinase (ERK) cascades. The p38 MAP kinase pathway is also activated in a JAK2-independent manner downstream of the leptin receptor. The NF-κB pathway is activated, predominantly *via* upstream Akt activation. Inhibitor studies have shown that the ERK, Akt, NF-κB and p38 pathways are all essential to the proliferative and anti-apoptotic effects of leptin. Co-ordinated activation of these pathways leads to enhanced expression of the cyclo-oxygenase-2 (*COX-2*) gene. This in turn enhances prostaglandin E2 (PGE2) production. PGE2 leads to by transactivation of the epidermal growth factor receptor (EGFR) and subsequent EGFR-dependant activation of the mitogen activated protein kinase cascades and late activation of c-Jun N-terminal kinase. As well as stimulating the initial steps in the pathway, leptin increases mRNA expression of the EGFR ligands and heparin binding EGF (HB-EGF) in EAC cells and immunoneutralisation of these growth factors blocks the proliferative effects of leptin, confirming their role in the pathway[95].

A separate JAK2-dependant pathway leading activation of signal transducer and activator of transcription 3 (STAT3) is also stimulated by leptin in EAC cells. Activated STAT3 also essential to the proliferation, anti-apoptotic and pro-invasive effects of leptin[98].

The experimental data show that leptin is able to stimulate malignant behaviour in Barrett's cells bearing the leptin receptor and thus may be a direct link between obesity and progression to EAC. As discussed previously, obesity seems to promote the development of Barrett's oesophagus and EAC through both reflux-dependent and independent mechanisms. Epidemiologically this combination of obesity and reflux is associated with a cancer risk significantly greater than either alone or when summated[21,31,43,64,65]. There are interesting parallels in the experimental cell models. The combination of exposure to leptin (as a model for obesity) and transient acid exposure (as a model of transient acid reflux) produced significantly greater (and synergistic) cell proliferation and reduction in apoptosis in EAC cell lines[56]. This acid-leptin combination resulted in synergistic activation of the Akt and ERK signalling cascades, without any further increases in leptin-receptor expression, COX-2 expression, PGE2 production and phosphorylation of either p38 MAP kinase or the EGFR[56]. *In vivo*, esophageal acid exposure enhances MAP kinase activation and mucosal proliferation[100] and increased AKT activation is associated with decreased apoptosis and progression to high grade dysplasia and cancer[101] Therefore it is possible that the continued exposure to the high levels of serum leptin seen in obese subjects enhances the response of Barrett's mucosa to even physiological acid reflux and promotes malignant change.

In addition to adipocytes, leptin is also synthesised and secreted by chief cells in the gastric body and can be detected in gastric juice. The function of this luminally-secreted leptin is unclear but it is possible it is a physiological regulator of mucosal integrity or nutrient absorption. Therefore esophageal mucosa is potentially exposed

to both circulating leptin and that in gastric refluxate, again suggesting some point of convergence between reflux-dependant and -independent mechanisms. Further studies are required into the possible role of gastric leptin however the presence of Barrett's oesophagus has been associated with increased levels of gastric fundic leptin[102].

ADIPONECTIN

Adiponectin, a 30 kDa protein, is the predominant protein secreted by adipocytes. Unlike leptin, adiponectin secretion falls as obesity increases and so obesity is characterised by relative adiponectin deficiency. The exact mechanisms causing this inverse relationship between fat mass and adiponectin secretion are unclear[103]. As might be expected, in general the effects of adiponectin are to oppose those of leptin and relative adiponectin deficiency has been implicated in the pathogenesis of the metabolic syndrome and its complications, including systemic inflammation. In general low systemic adiponectin levels have been associated with an increased risk of many cancers (including breast, colorectal, prostate, endometrial and gastric)[104]. Comparison between studies is complicated by the various circulating forms of adiponectin, which may have different biological actions and are detected in different assays[105]. Adiponectin is secreted as a full-length monomer (f-adiponectin) than then aggregates into both low- molecular and high-molecular weight oligomers. A truncated form (globular adiponectin (g-adiponectin)) is also found, this is at least partly formed by breakdown of full-length adiponectin by enzymes released in inflammation and circulating levels may not accurately reflect tissue levels of g-adiponectin[106]. There at two specific cell surface adiponectin receptors: AdipoR1 which appears to be relatively globular adiponectin specific and AdipoR2 which has equal affinity for globular and full length adiponectin[107]. Adiponectin may also be able to exert cellular effects by binding to, and inhibiting the action of, HB-EGF[108].

Data are available to support relative adiponectin deficiency in the promotion of EAC (Figure 2). AdipoR1 and AdipoR2 are expressed on both non-dysplastic and neoplastic Barrett's epithelium[97,109,110]. Circulating adiponectin levels have been shown to be inversely associated with the risk of both Barrett's oesophagus[83,93,111], and erosive oesophagitis[112]. Increased levels of low-molecular weight adiponectin and a high low-molecular weight/total adiponectin ratio have been shown to be independently associated with a reduced risk of developing Barrett's esophagus in patients with gastro-esophageal reflux disease[113].This relationship has not been seen in all studies[92]. Perhaps more convincingly low serum levels of adiponectin have been reported to be an independent risk factor for neoplastic progression in a cohort of Barrett's patients[43].

In a variety of experimental studies adiponectin has been shown to exert anti-cancer effects in Barrett's cancer cell lines. Adiponectin inhibits leptin-induced proliferation, inhibits leptin-induced invasion and migration and ameliorates the anti-apoptotic effect of leptin. Inhibition of AdipoR1 with RNA interference prevented these effects. Downstream of AdipoR1, these effects are mediated by 5'-AMP activated kinase (AMPK), which ultimately leads to blunting of leptin signalling *via* inhibition of the Akt pathway[110]. Further detailed studies have shown these inhibitory effects are mediated by the activation of the relatively non-specific protein tyrosine phosphatase PTP1B. Adiponectin leads to increases in both PTP1B mRNA and protein expression and also a separate activation of PTP1B enzyme activity. Activation of this tyrosine phosphatase inhibits signalling *via* the leptin receptor. These experimental models provide a basis to explain how leptin, adiponectin and acid may interact at the cellular level to promote either the promotion or persistence of Barrett's epithelium or malignant behaviour in cancer cells and how obesity can remotely influence the risk so EAC[98]. Although speculative at this stage, this potential mechanism of adiponectin *via* PTP1B could have wider importance. PTP1B is a relatively non-specific phosphatase and would also be expected to inhibit signalling *via* other pathways that are believed to be important in driving malignant behaviour in Barrett's epithelium, such as EGFR ligands, IL-6 and bile acids or even those pathways leading to cdx2 expression, which are believed to be central to the development of the Barrett's phenotype[41,114,115]. Hence relative adiponectin deficiency in obesity could contribute to the development and progression of Barrett's esophagus at many steps.

The different types of adipose tissue have different hormonal effects. As discussed previously EAC and Barrett's are most clearly associated with abdominal rather than general obesity[31]. Even within this abdominal obesity there are variable contributions from the separate visceral and subcutaneous fat tissues. More specifically, excess visceral fat being specifically associated with Barrett's esophagus. Gluteofemoral fat ("hips") (which is subcutaneous fat) does not seem to be a specific risk factor of BE and may even be protective[34] It is thought gluteofemoral and subcutaneous fat is even less metabolically active and has less effect on progression of Barrett's oesophagus. In light of this, it is believed that visceral, rather than subcutaneous, fat is usually the predominant source of circulating adiponectin[116-118] and this might explain how reduction in adiponectin secretion from visceral fat probably specifically contributes to the Barrett's-carcinoma sequence.

IMPLICATIONS FOR THERAPY

The fact that obesity is a risk factor for both BE and EAC is established. This is already being translated into the clinical arena: for example the British Society of Gastroenterology guidelines now suggest that screening and case finding for Barrett's esophagus be considered

in males with reflux symptoms and at least two other risk factors (Caucasian, obesity, smoker), this has the advantage of detecting premalignant cases of Barrett' s esophagus that may be amenable to surveillance and endoscopic therapies if required[119]. A broader question is how may our understanding of the pathophysiological links between obesity and EAC be translated into useful therapeutic gains for prevention or treatment?

The mechanisms linking obesity and esophageal are undoubtedly complex and likely multifactorial and are likely to differ depending on the histological stage of the esophageal mucosa. Experimental and epidemiological studies support a role of the adipokines leptin and adiponectin in the progression to EAC but further mechanistic and clinical studies are still required. At the present time, these pathophysiological insights have suggested several new areas of therapy.

Although it is accepted that gastro-esophageal reflux plays a central role in the pathogenesis of BE and EAC and appears to accentuate the risks associated with obesity, profound acid suppression with either proton pump inhibitors or anti-reflux surgery have not conclusively been shown to have chemopreventative effects. The large United Kingdom AspECT trial comparing placebo, aspirin and standard- and very high dose-esomeprazole in a randomized trial may provide clarity on this issue when data become available[120].

At both a population and individual level weight loss with dietary and behavioural modifications remains the first line approach for obese patients. Gastric bands tend to accentuate reflux and for those patients with reflux symptoms and significant obesity[121], a Roux-en-Y gastric bypass appears to be the preferable procedure, although it cannot be advocated purely to prevent esophageal cancer. Interestingly, as well as a reduction in body mass and visceral fat, and reducing symptoms from gastro-esophageal reflux, this procedure is associated with potentially beneficial metabolic effects including higher serum adiponectin[39,122].

There may yet be some developments in therapies aimed to improve the metabolic/endocrine profile of adipose tissue that may translate into useful clinical interventions. Antagonists of CB1 receptors, such as rimonabant reduce visceral fat[123] and the PPAR-α agonists such as rosiglitazone enhance adiponectin release from visceral fat[118]. Unfortunately at the present time the adverse effects; psychiatric problems with rimonabant and bladder cancer and the increased cardiovascular mortality with PPAR-α agonists preclude their wider use. A variety of other agents have been shown to usefully increase serum adiponectin levels: these include PPAR-α agonists, inhibitors of the renin-angiotensin system, calcium channel modulators and some beta-receptor antagonists[124] and various phyochemical such as catechin[125]. All these deserve further study, although at this time, data are limited and these drugs and their effect on adipokine profiles have not been investigated in the context of esophageal disease[124]. Preclinical development of adiponectin-analogues[126] and leptin-receptor antagonists[127] is continuing but these are some way off clinical use.

Metformin seems to have some potential as a chemopreventative agent in the context of obesity-associated EAC. Metformin is a direct activator of AMPK kinase and exerts potentially useful anti-cancer effects[128]. In Barrett's cell line studies, the inhibitory effects of adiponectin are also mediated *via* activation of AMPK[110]. In case-control studies, metformin use is associated with a reduced incidence of many cancers including esophageal cancer[129]. Metformin is inexpensive, has a low incidence of side effects and could be promising chemopreventative agent, although more studies specifically in EAC are needed.

There appear to be more data to recommend aspirin and statins (HMG-CoA reductase inhibitors) as the most appropriate potential chemopreventative agents to reduce the incidence of EAC associated with, or indeed without obesity. Several lines of experimental data show that cyclooxygenase inhibitors, such as aspirin, reduce malignant behaviours such as proliferation and apoptosis-inhibition in EAC and non-neoplastic Barrett's cells lines. Non-specific and COX-2 selective inhibitors block the effects of leptin in cell line models[130-132]. Definitive conclusions on the preventative effects of aspirin may have to wait until the UK AspECT trial has reported[120]. Observational studies and meta-analyses show that aspirin use is associated with a reduced incidence of both Barrett's esophagus and EAC[133,134]. Statins exert potent anti-cancer effects in EAC cells line models. By inhibiting the post-translational modification (prenylation) of small signalling G protein of the Ras/Rho family and so ameliorating pro-carcinogenic signalling from growth factor receptors, statins inhibit cell growth and induce apoptosis[135]. Experimentally the effect of inhibition of the COX-2/PGE2 pathway, by using a variety of small molecule COX-inhibitors, inhibition of microsomal PGES-1 or RNA interference, and the effect statins were additive[131,135]. A similar magnitude of reduced risk has been reported in two separate meta-analyses of Barrett's cohorts, where the combination of COX-inhibitor and statin was associated with a 85% reduction in EAC incidence[133,136]. Statins may also have beneficial effects by increasing increase serum adiponectin levels[124]. It is probably premature to advocate aspirin and statin therapy as primary preventative therapy for all. It is essential to consider that cardiovascular disease and not EAC is predominant cause of death in Barrett's cohorts and hence that statins and aspirin should be utilised for the beneficial effects on circulatory diseases pending further clarification of the chemopreventative actions[131].

CONCLUSION

Overall a large amount of epidemiological data shows that obesity is likely to be causally associated with esophageal adenocarcinoma. This cancer is strongly associated with an increase in BMI, in fact more so than for other cancers. There are also strong associations between mea-

sures of adiposity and gastro-esophageal reflux including the more serious sequelae, reflux esophagitis and Barrett's esophagus. Abdominal, and in particular visceral, obesity is likely to play a key role in its pathogenesis though both reflux-dependent and -independent mechanisms. Leptin and adiponectin are adipokines secreted by visceral fat cells and both an increased serum leptin decreased serum adiponectin have been reported to be risk factors for progression to EAC. Experimentally, leptin enhances, and adiponectin inhibits malignant behaviour in Barrett's cell lines, consistent with these mediators having a direct role in the pathogenesis of EAC. No specific chemopreventative strategies are of proven benefit, but appropriate weight loss in overweight subjects seems appropriate. Aspirin and statins seem to have the most potential as chemopreventative actions and should be utilized in patients with Barrett's esophagus according to the cardiovascular risk profile.

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REVIEW

Intestinal microbiota: The explosive mixture at the origin of inflammatory bowel disease?

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Abstract

Inflammatory bowel diseases (IBDs), namely Crohn's disease and ulcerative colitis, are lifelong chronic disorders arising from interactions among genetic, immunological and environmental factors. Although the origin of IBDs is closely linked to immune response alterations, which governs most medical decision-making, recent findings suggest that gut microbiota may be involved in IBD pathogenesis. Epidemiologic evidence and several studies have shown that a dysregulation of gut microbiota (*i.e.*, dysbiosis) may trigger the onset of intestinal disorders such as IBDs. Animal and human investigations focusing on the microbiota-IBD relationship have suggested an altered balance of the intestinal microbial population in the active phase of IBD. Rigorous microbiota typing could, therefore, soon become part of a complete phenotypic analysis of IBD patients. Moreover, individual susceptibility and environmental triggers such as nutrition, medications, age or smoking could modify bacterial strains in the bowel habitat. Pharmacological manipulation of bowel microbiota is somewhat controversial. The employment of antibiotics, probiotics, prebiotics and synbiotics has been widely addressed in the literature worldwide, with the aim of obtaining positive results in a number of IBD patient settings, and determining the appropriate timing and modality of this intervention. Recently, novel treatments for IBDs, such as fecal microbiota transplantation, when accepted by patients, have shown promising results. Controlled studies are being designed. In the near future, new therapeutic strategies can be expected, with non-pathogenic or modified food organisms that can be genetically modified to exert anti-inflammatory properties.

Key words: Intestinal microbiota; Inflammatory bowel diseases; Probiotics; Prebiotics; Symbiotics

Core tip: This paper focuses on the scientific scenario regarding the potential function of gut microbiota in inflammatory bowel diseases (IBDs). Epidemiologic findings suggest that the heterogeneity and disruption of gut microbiota can be significant in modulating and addressing the immune reactions underlying IBD pathogenesis. Traditional or innovative manipulation strategies of gut microbiota may be possible future treatment options for the management of these disorders.

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INTRODUCTION

Inflammatory bowel diseases (IBDs) are lifelong chronic disorders arising from interactions among genetic, immunological and environmental factors[1].

Technological advances have allowed novel predictive factors to be assessed, that can identify the disease at an early stage and provide an accurate diagnosis long before the onset of clinical manifestations[2]. Recent findings suggest that, in addition to genetic and environmental factors, interactions with the gut microbiota may play a relevant role in a "perfect storm" driving the pathogenesis of IBDs[3].

MICROBIOTA AND IBD

The human intestinal tract includes several multifaceted microbial populations with an essential function in general health. The human gut contains, in the assortment of 1000 bacterial species, 100-fold more genes than the human genome. The new high throughput sequencing technologies, the presence of 16S rRNA genes in the gut bacterial composition, as well as recent non genomic techniques, have well defined the function of gut microbiota in some human diseases[4,5].

Although the microbiota of the colon is apparently similar in different people, there are marked variations between individuals in bacterial populations of a single species. It has been demonstrated that an increase in biodiversity requires a different metabolic homeostasis and structural stability, while a reduction, due to age, illness or antibiotics, reduce the capacity of the intestinal environment to fight infecting pathogens[6,7]. In fact, epidemiological evidence and experimental studies have suggested that alterations in the gut microbiota (*i.e.*, dysbiosis) can be relevant in intestinal conditions such as chronic IBD[8].

Clinical evidence confirms the role of microbiota in IBD, and an abnormal microbial composition in IBD has been amply demonstrated. The most common site of IBD is the colon, where the highest intestinal bacterial concentrations are found. Additionally, fecal stream diversion can prevent and treat Crohn's disease (CD) and pouchitis. Finally, many studies have shown that antibiotics and probiotics improve the histological, endoscopic and clinical picture[9]. Despite this evidence-based findings, there are still some major unexplained points such as the IBD response to immunosuppressive therapy or the protective role of poor hygienic conditions, which do not appear likely to be related to the microbial state[10].

Animal studies

It is known that the non-pathogenic microbiota controls bowel immunity, but interactions in the gut with host microbes can be bidirectional. The mucosal immune system can be affected by the pro-inflammatory potential of abnormal growth of microbiota elements, which ultimately determine or influence an inflammatory reaction and induce the possibility of development of illness. Several animal studies have shown that this interaction is possible and can induce colitis.

Studies in germ-free interleukin 10-deficient (IL-10$^{-/-}$) mice, that fail to acquire spontaneous colitis and immune activation, support this hypothesis[11]. Indeed, some studies show that, regardless of the background strain of these animals, the onset and degree of spontaneous colitis depends on the composition of the enteric gut microbiota[11,12]. Penetrance of colitis increases to nearly 100% when the immune system response is characterized by a T-helper 1 (Th1) interferon (IFN)-γ reaction[12].

Therefore, in this model of colitis, it has been demonstrated that the disease may show different characteristics and distribution based on the intestinal bacteria present. Furthermore, in IL-10-/- germ-free mice, bacterial colonization of non-pathogenic bacteria such as *Escherichia coli* (*E. coli*) or *Bilophila wadsworthia* provokes different types of colitis[13]. In particular, *Bilophila wadsworthia* produces a low grade colitis involving the distal colon, associated with an exclusively Th1-mediated immune response. In contrast, *E. coli* leads to an early (3-wk) development of mild-to-moderate inflammation that is more severe in the cecum. In the same study, *Bacteroides vulgatus*, but not *E. coli*, provokes mucosal inflammation of the colon in HLA-B27 transgenic mice without bone marrow involvement as in transplanted CD3 transgenic mice[13].

Finally, novel experimental evidence demonstrated that *Klebsiella* may provoke moderate pancolitis while *Bifidobacterium animalis* could cause a mild degree of inflammation in the distal colon and duodenum[14,15].

Human studies

A few studies in humans have suggested that IBD patients have an altered balance of intestinal microbiota in the active phase. Bacterial 16S rRNA gene examination did not show relevant differences in bacterial constitution in the intestinal mucosa of CD and ulcerative colitis (UC) patients. Moreover, in UC patients, a decrease in bacterial load was observed even if it was not significant when compared to that of CD patients[16-18].

Another interesting finding, a thinner and less sulphated mucus in patients affected by UC, has been demonstrated and may account for an increased number of bacteria colonizing the mucosa[19,20]. Indeed, a poor mucus layer with a microbiota overgrowth could enhance the presentation of bacterial antigens to the immune system of the gut mucosa. In UC patients, the colonic surface and inflamed areas are colonized by a broad variety of bacteria. For example, in UC specimens *Clostridium histolyticum* and *lituseburense* accounted for 21% of the microbiota. *Enterobacteriaceae* such as *Escherichia* and *Klebsiella* have also been considered to be implicated in the pathogenetic mechanism of UC. Indeed, their aptitude to adhere to enterocytes, allowing them to penetrate the mucosal layer and deliver enterotoxins, might account for this hypothesis[21,22].

Genetics in IBD pathogenesis

The interaction between genetic factors, and a dysregulated response of the immune system to bacterial antigens are still strongly supported hypotheses in the pathogenesis of IBD. Indeed, genome-wide association studies (GWAS) showed that several genes were associated with

IBD susceptibility[23]. These genes, risk factors for CD and UC, encode for proteins that may regulate the microbiota (NOD2/CARD15) or may control host responses (IL-12-IL23R pathway or autophagy)[24,25], and constitute a barrier function notably for UC[26].

One of these proteins, NOD2, may be crucial for distinguishing between non-pathogenic and pathogenic organisms; indeed, it initiates signal transduction thus promoting NFκB translocation into the nucleus, where the expression of specific genes determinates the response of primary and adaptive immune mechanisms[27-29].

The multifunctional genetic linkage of NOD2/CARD15 is demonstrated by the protein's ability to identify bacterial muramyl-dipeptide and by its impact on the homeostasis of non-pathogenic bacteria, regulatory T cells (Tregs), and viral identification by immune system[24]. Although NOD2 homozygosity may carry a 20-fold increased risk for CD, notably in the ileal location, less than 20% of patients affected by CD are homozygous for NOD2[30,31]. So, while these studies and GWAS have provided important details about IBD pathogenesis, investigations on the distribution of genetic variants in different populations poorly explain the large discrepancies in IBD prevalence between different geographic areas as well as the increasing incidence of IBDs in Western countries over the past 5 decades[2].

The evidence strongly supports that IBDs are polygenetic disorders and their heterogeneity relates to the complexity of their genetic background as well as to different lifestyle and environmental factors, including variations in microbiota composition.

Environmental triggers

It is known that nutrition, medications (NSAIDs) and smoking affect the composition of the gut microbiota and it is known that changes in this multifaceted structure are contributing factors in the origin of some disorders, including IBDs.

Smoking is a relevant risk factor in CD pathogenesis[32-36]. Indeed, it may alter the intestinal microbiota and its cessation may further modify intestinal microbial composition. Indeed, simultaneous increased *Firmicutes* and *Actinobacteria* and decreased *Proteobacteria* and *Bacteroidetes* characterize smoking cessation; in contrast, the composition of the flora in continuous smokers and non-smokers remains stable[37].

Many studies have reported a modification of the gut microbiota composition in populations migrating from developing to developed countries[38]. In these subjects, diet, family size, antibiotic consumption, urbanization, reduced parasitism, and a reduction in exposure to childhood infections, such as hepatitis A and *Helicobacter pylori,* are associated with changes in the microbiota.

Neonates show a sterile or, at least, a very low microbial load in the intestine[39]. Bacterial strains colonize the infant bowel after delivery according to various factors, such as method of delivery, breast- or bottle-feeding, and antibiotic administration[40]. There is early colonization of *Lactobacillus* and *Prevotella* after vaginal delivery and greater colonization of *Firmicutes* in neonates delivered by cesarean section, that predisposes to a greater susceptibility to some pathogens and a higher risk of atopic disease[41,42]. Therefore, growth from newborn to early childhood and finally adulthood is associated with changes in the gut microbiota, featuring a reduction in *Lactobacillus* and *Bifidobacteria* and an increase in *Firmicutes, Clostridia* and *Bacteroides* species, that may lead to a high risk of allergic and immunological diseases[43]. This raises the hypothesis that a decreased biodiversity within non-pathogenic microbiota, with an altered immunity maturation, could negatively influence the immune recognition and activation, and thereby confer a risk for developing IBD in adulthood[38].

Regarding the impact of a high-fat dietary intake on the non-pathogenic microbiota, it has been demonstrated that it can radically remodel the intestinal microbiota[44,45]. Moreover, there is evidence that non-absorbed carbohydrates (inulin and fructooligosaccharides) promote the growth of beneficial species, supplying a substrate for the production of short-chain fatty acids (SCFAs)[46].

Recently, novel studies have focused on the role of NSAIDs in inducing and maintaining mucosal damage, thus contributing to the genesis of IBD. In particular, several studies demonstrated that NSAIDs were able to cause injury by means of microbiota modulation[47]. NSAIDs, indeed, can promote the overexpression of proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and IFN-γ through changes in the microbiota[48], and further allow bacterial translocation through the intestinal barrier. This hypothesis is confirmed by evidence that the levels of such proinflammatory cytokines are significantly increased in IBD patients.

Microbiota and IBDs: Comments on the literature

There can be no doubt, in view of all the experimental data, that the microbiota can be considered a key factor in the origin of IBDs and not a bystander. Studies performed on animal models provide strong evidence for a primary role played by microbiota in IBDs but human studies do not fully support this pathogenic hypothesis owing to the lack of sufficient scientific proof. For instance, it is well-known that, in CD, the entire alimentary tract from the oral cavity to the anus may be involved, but no data from human studies are available on this topic. Conversely, animal studies have demonstrated that the microbiota composition may influence the onset of IBDs in a selected part of the digestive system. El Aidy *et al*[49] investigated the responses of the jejunal mucosa to bacterial colonization in germ-free mice, showing a consequent shift to anaerobic metabolism, a condition that may strongly influence mucosal oxygenation in IBD. Moreover, in an experimental model of small bowel CD, a single strain of *E. coli* (LF82) has been demonstrated to stimulate the production of proinflammatory cytokines, an effect that was counteracted by lactoferrin, another microbial product[50].

There has been much discussion as to whether infec-

Table 1 Antibiotic therapy in inflammatory bowel diseases

Ref.	Year	Antibiotics	Duration	Result
Crohn's disease-primary therapy				
Ursing *et al*[53]	1982	Metronidazole 800 mg/d	16 wk	No difference from sulfasalazine
Sutherland *et al*[54]	1991	Metronidazole 10 or 20 mg/kg	16 wk	Superior to placebo (↓ CDAI), no difference in remission
Colombel *et al*[55]	1999	Ciprofloxacin 500 mg 2 × d	6 wk	No difference from mesalamine
Arnold *et al*[56]	2002	Ciprofloxacin 500 mg 2 × d	6 mo	Superior to placebo (CDAI)
Prantera *et al*[57]	1996	Ciprofloxacin 500 mg 2 × d + metronidazole 250 mg 4 × d	12 wk	No difference from prednisolone
Greenbloom *et al*[58]	1998	Ciprofloxacin 500 mg 2 × d + metronidazole 250 mg 3 × d	10 wk	Uncontrolled, 68% remission
Leiper *et al*[59]	2000	Clarithromycin 250 mg 2 × d	4 wk	Uncontrolled, 64% response, 48% remission
Steinhart *et al*[60]	2002	Ciprofloxacin 500 mg 2 × d + metronidazole 250 mg 3 × d	8 wk	No improvement over budesonide alone (33% *vs* 38% remission)
Crohn's disease-prevention of postsurgical relapse				
Rutgeerts *et al*[61]	1995	Metronidazole 20 mg/kg	12 wk	↓ clinical relapse 1 yr *vs* placebo
Rutgeerts *et al*[62]	2005	Ornidazole 1 g/d	52 wk	↓ severe endoscopic relapse *vs* placebo
Ulcerative colitis-primary therapy				
Turunen *et al*[63]	1999	Cipro 500 mg 2 × d	6 mo	Superior to placebo
Mantzaris *et al*[64]	1997	Cipro 500 mg 2 × d	6 mo	No benefit *vs* placebo
Casellas *et al*[65]	1998	Amoxicillin 1 g/ Clavulanic acid 250 mg	5 d	↓ mucosal IL-8 and eicosanoids *vs* placebo
Turner *et al*[66]	2014	metronidazole, amoxicillin, doxycycline (Paediatrics)		Remission (46.6%)
Pouchitis				
Shen *et al*[67]	2001	Metronidazole 20 mg/kg or Cipro 500 mg 2 × d	6 mo	Both effective, Cipro > metronidazole
Gionchetti *et al*[68]	2000	Cipro 500 mg 2 × d and Rifaximin 1 g 2 × d	5 d	89% response, 33% remission, uncontrolled

tious factors could be a trigger for IBD. No evidence is available from human studies, but animal models offer interesting insights. Couturier-Maillard *et al*[51] demonstrated that microbiota transplantation from healthy wild-type mice may reduce the IBD risk in Nod2-deficient mice and lead to long-term alterations in the gut microbiota. On the other hand, disease risk was promoted in wild-type mice that were recolonized with dysbiotic fecal microbiota from NOD2-deficient mice. In conclusion, animal models must be seen only as a starting point for microbiota investigation in humans, and the main lesson that we can deduce is that an imbalance of bacterial species is one of the main reasons that can explain the different types of colitis induced by the effect of different bacteria.

PHARMACOLOGICAL MANIPULATION OF MICROBIOTA IN IBDs

Antibiotics

Antibiotics are known to have an important role in the management of septic complications of IBD, *e.g.*, intra-abdominal and perianal abscesses and fistulae of CD, superinfections, and post-surgical wound infections. Nonetheless, treatment with antibiotics for active luminal CD and UC is not widely accepted as a first-line choice. Although the use of antibiotics against pathogenic bacteria is proven and based on reliable evidence of experimental enterocolitis and IBD, there are some clinical trials that do not sufficiently support the efficacy of these drugs in patients affected by IBD[52].

The most representative published studies are summarized in Table 1[53-68]. Metronidazole, ciprofloxacin, or the contemporary use of these agents are useful in Crohn's colitis, ileocolitis and pouchitis, but not in disease confined to the ileum. They are recommended for pouchitis in the European Crohn's and Colitis Organisation statements, which also indicate that ciprofloxacin appears to have fewer adverse effects (statements 8C, 8D)[69].

Probiotics

Probiotics are viable microorganisms that have been cultured from foods, in particular milk. Various species and bacterial strains that have been used in IBD clinical trials, are believed to have a potential beneficial role. The most evaluated probiotics are *E. coli Nissle*[70], *VSL#3* mixture (four strains of *lactobacilli*, three strains of *bifidobacteria*, and one strain of *Streptococcus salivarius thermophilus*)[68,71-73], *BIO-THREE* mixture (*S. faecalis, C. butyricum,* and *Bacillus mesentericus*)[74], a mixture of *L. rhamnosus* and *L. reuteri*[75], *L. rhamnosus GG*[76], Yakult strains of *Bifidobacterium brevis, Bifidobacterium bifidum and L. acidophilus*[77]. Recently, advanced genetic engineering has produced modified species that are able to produce immunosuppressive molecules such as IL-10[78].

These studies have shown that probiotic supplementation can re-establish bacterial homeostasis in the intestine and downregulate gut inflammation that is characteristic of IBD patients, thus modulating the inflammatory/anti-inflammatory balance. A reduction in the number of microbiome elements was also found. Indeed, the administration of probiotics can normalize

Table 2 Probiotic therapy in inflammatory bowel diseases

Model	Probiotic	Effect
Trinitrobenzene sulphonic acid or dinitrobenzene sulphonic acid	*Bi. infantis*	No effect
	L. acidophilus, L. casei and *Bi. animalis*	Reduced inflammation
	VSL#3	No effect
	Lactobacillus GG	No effect
	L. plantarum 299	No effect
	VSL#3 (DNA, subcutaneously)	Reduced inflammation
Iodoacetamide	VSL#3	Reduced inflammation
	Lactobacillus GG	Reduced inflammation
Acetic acid	*L. rhamnosus GG*	No effect
	L. reuteri R2LC	Reduced inflammation
	L. reuteri R2LC	Reduced inflammation
Dextran sodium sulphate	VSL#3 (irradiated and DNA*)	Reduced inflammation
IL-10 knockout mice	*L. salivarius* 118 (subcutaneously)	Reduced inflammation
	L. salivarius	Reduced inflammation
	Bi. infantis	Reduced inflammation
	L. plantarum 299V	Reduced inflammation
	VSL#3	Reduced inflammation
	L. salivarius	Reduced inflammation
	L. reuteri	Reduced inflammation
	VSL#3 (DNA, subcutaneously)	Reduced inflammation
E. coli-induced colitis in IL-2 knockout mice	*B. vulgatus*	Reduced inflammation
B. vulgatus-induced colitis	*Lactobacillus GG*	Prevented recurrent colitis
	L. plantarum 299*V*	No prevention of recurrent colitis

altered intestinal microbiota in IBD patients, and increase protective species by reducing the pathogen load, positively affecting intestinal permeability, balancing local immune response, producing beneficial substances, and disintegrating pathogenic antigens in the intestinal lumen[79].

In animal models (Table 2), *Lactobacilli* and *Bifidobacteria* reduced the severity of experimental colitis in IL-10 knockout mice[80,81]. In another study *L. plantarum* prevented colitis onset in HLA B27 transgenic rats. This and other reports confirm the protective effects of several probiotics in selected hosts and special inflammatory conditions. Therefore, in experimental colitis induced in B27 transgenic rats, which had remission with broad-spectrum antibiotics, probiotics prevented recurrence of the colitis. However, probiotic treatment alone was unable to produce remission of the induced disease[82].

The beneficial effect of probiotics was demonstrated in rats with colitis induced by instillation of 4% acetic acid, which causes altered intestinal permeability. In particular, after 4 d of acetic acid treatment the activity of myeloperoxidase (MPO) showed a 3-fold increase, in parallel with a 6-fold increase in mucosal permeability in the colonic samples. The use of *L. reuteri R2LC,* after acetic acid administration, reduced the morphological score, MPO activity, mucosal permeability, and prevented the onset of colitis[83].

In human studies, 9-mo daily use of a probiotic formula, *i.e.*, *VSL#3,* was effective in preventing the relapse of chronic pouchitis after remission induced by antibiotics[68]. Another investigation replicated the same results, and, in addition, showed a decreased frequency of pouchitis when *VSL#3* was given after pouch closure[84].

In cases of mild-to-moderate active UC treated with probiotics, there was an improvement in clinical severity, a reduction in relapses, and induction of remission. Moreover, these findings were accompanied by high histological scores and increased levels of fecal butyrate and other SCFAs[73-77].

Studies in UC patients found that the prevention of flare-ups by probiotics was associated with inactivation of NF-κB, downregulation of TNF-α and IL-1β, and a simultaneous increase in anti-inflammatory cytokines, such as IL-10[85]. Few data are available about the mechanism by which probiotics could modify the composition of the resident microbiota, even though it has been hypothesized that they might increase the load of *Lactobacilli* and/or *Bifidobacteria*[74,85].

On the other hand, clinical trials with the use of probiotics in CD, are less concordant than in UC. Malchow[86] found that *E. coli Nissle* was more effective than placebo in preventing relapse of CD in the remission phase induced by conventional therapy, but supplementation of probiotics was found to be ineffective in prolonging remission after the administration of *L. johnsonii LA1* following surgical resection[87,88]. Similarly, a study of Prantera *et al*[80] did not demonstrate any benefit by 1 year-long *Lactobacillus GG* consumption in the prevention of post-surgical clinical or endoscopic relapses in the neo-terminal ileum.

As reported above, Butterworth *et al*[89] evaluated 12 potentially relevant studies of the efficacy of probiotics in CD, even though 11 did not fulfill the inclusion criteria. In the only study satisfying the stated criteria, patients with moderately active CD received *L. rhamnosus* GG for 6 mo without obtaining an improvement.

Prebiotics

Prebiotics are dietary supplementations, usually non-digestible glycides, which are energy substrates for protective intestinal organisms. Lactosucrose, fructo-oligosaccharides, inulin, bran, psyllium, and germinated barley extracts promote *Lactobacilli and Bifidobacteria* growth, thus inducing SCFA production, in particular butyrate[90-92]. Therefore, these substances are able to re-establish the optimal beneficial/pathogen bacteria ratio in IBD patients. These physiological dietary supplements increase the protective lactic acid bacilli load, with a consequent inhibition of harmful species by decreasing the luminal pH, reducing epithelial adhesion, and producing bactericidal molecules. Animal studies showed a protective effect in rat colitis models (Table 3)[93,94]. Several small controlled studies but only a few randomized controlled

Table 3 Inflammatory bowel diseases prebiotic therapy

Model	Prebiotic	Effect
Trinitrobenzene sulphonic acid	Fructo-oligosaccharide	Reduced inflammation
	Galacto-oligosaccharide	No effect on inflammation
Dextran sodium sulphate	Fructo-oligosaccharide	No effect on inflammation
	Resistant starch	Reduced inflammation
	Germinated barley foodstuff	Reduced inflammation
	Germinated barley foodstuff	Reduced inflammation
	Inulin	Reduced inflammation
	Germinated barley foodstuff	Reduced inflammation
IL-10 knockout mice	Lactulose	Reduced inflammation

trials (RCT) in IBD patients have been performed, fewer than the studies with probiotics.

Interestingly, Welters *et al*[95] carried out a clinical trial in 20 patients with an ileal pouch-anal anastomosis who consumed 24 g of inulin or placebo daily for 3 wk. The pH, short chain fatty acids, microflora, and bile acids were determined in the stools, while the inflammatory status was evaluated by clinical, endoscopic and histological parameters. It was proven that the treatment enhanced butyrate levels, reduced pH, and reduced the number of *Bacteroides fragilis* as well as fecal concentrations of secondary bile acids. These findings were accompanied by a reduction in inflammation in the ileal reservoir mucosa.

In another open-label study, 10 patients with active ileocolonic CD were enrolled to receive a daily 15 g dose of fructo-oligosaccharides (FOS) for 3 wk. The Harvey-Bradshaw index was chosen to assess the disease activity, and fluorescence *in situ* hybridization was used to measure *Bifidobacteria* in stools; flow cytometry of dissociated rectal biopsies evaluated mucosal dendritic cell, IL-10 and TLR expression. The results of this study were promising: the use of FOS resulted in a significant reduction in the Harvey Bradshaw index, and a significant increase in fecal *Bifidobacteria* concentrations. The percentage of IL-10 positive dendritic cells was increased from 30% to 53%. Moreover, an increase in the percentage of dendritic cells expressing TLR2 and TLR4 was found (from 1.7% to 36.8% and from 3.6% to 75.4%, respectively)[96].

Symbiotics

Probiotic therapy can potentially be improved by simultaneous administering of prebiotics (non-digestible and non-absorbable carbohydrates) that enhance probiotic proliferation in the gut. This mixture is referred as a symbiotic. The main benefit of symbiotic formulation is that a prebiotic constituent could positively modulate the increase in local microbiota, which is further regulated by the probiotic component of the synbiotic formulation. In animal models, Schultz *et al*[97] evaluated the effect of a symbiotic preparation composed of a probiotic combination of *Lactobacilli, Bifidobacteria* and inulin (SIM) in HLA-B27-beta(2)-microglobulin transgenic rats affected by severe colitis. After 4 mo of SIM treatment, the colonic disease showed histological improvement and, furthermore, there was an alteration in the microflora profile, with an increased variety, and specifically, increased growth of *Bifidobacterium animalis* compared with untreated rats.

A few well conducted studies have supported the usefulness of symbiotic supplementation. Furrie *et al*[98], in a double-blinded RCT, developed a symbiotic called Synergy 1, made up of a combination of a probiotic (*Bifidobacterium longum*) and a prebiotic (inulin-oligofructose), which provided a metabolic substrate for the *Bifidobacterium* strain, and obtained promising results in UC patients.

Fecal transplantation

A novel treatment for IBD is fecal microbiota transplantation (FMT). FMT consists of extracting gastrointestinal microbiota from a healthy donor, which is then instilled *via* an enema through a liquid stool suspension. FMT has recently gained ground as a therapy for refractory and/or recurrent *C. difficile* infection[99-102].

In a recent systematic review conducted by Anderson *et al*[103], following Cochrane and PRISMA recommendations, 5320 articles on FMT in patients with IBD were identified. Seventeen articles were selected, including reports on FMT given in single cases to treat IBD, and in the management of infectious diarrhea in IBD. The 17 trials encluded 41 subjects followed up for 2 wk-13 years. FMT was able to produce a reduction in symptoms in most of the IBD patients, allow an interruption in IBD medication, and result in disease remission. In those patients who experienced a simultaneous *C. difficile* infection, complete eradication of the bacterium was achieved. Even though this procedure may face difficult acceptance by patients, the review describes promising results.

Despite insufficient data on FMT in IBD, this procedure is potentially an effective and safe treatment; it may be suggested for subjects who failed conventional treatments. It is necessary to perform new well-designed and randomized trials to enrich the data about FMT in IBD to: (1) evaluate safety and success rate; and (2) to standardize protocols. Without these considerations, FMT could not become a standard part of clinical therapy[103].

CONCLUSION

Patients affected by IBDs, either UC or CD, suffer from a heterogeneic entity whose pathogenic etiology must be explored in the context of a "multihit" phenomenon that precipitates the disease through a multifactorial platform resulting from interactions among genetic, immunological and environmental triggers. Although the microbiota may well play a crucial role in the origin of IBD, up-to-date therapeutic strategies have a primary purpose of suppressing the host response, and so a significant fraction of patients fail to accomplish sustained remission.

While novel techniques in molecular biology and engineering have enabled further discoveries about the gut microbiota, the relationship between intestinal microbiota

and IBD has not yet been completely clarified. A better understanding of the role that some bacterial species play in IBD pathogenesis is essential in order to develop appropriate management strategies.

The possibility of modulating our gut flora by interventions on microbial composition and the correct timing of this operation have important implications on efforts to improve gastrointestinal health. Nevertheless, microbiology should support, but not replace, the genetics of IBD, and meticulous typing of the intestinal microflora should soon take a decisive place in its complete characterization in order to explore the relationship between genes and the environment in health and disease. Finally, future research in microbial intervention needs to be directed towards two areas: (1) improvements in strain selection with the goal of realizing new screening procedures for a better understanding of the mechanisms of action, and ensuring adequate efficacy; (2) a new therapeutic strategy with non-pathogenic organisms of alimentary origin that can be genetically modified with the aim of producing anti-inflammatory substances.

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REVIEW

Patterns of airway involvement in inflammatory bowel diseases

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Abstract

Extraintestinal manifestations occur commonly in inflammatory bowel diseases (IBD). Pulmonary manifestations (PM) of IBD may be divided in airway disorders, interstitial lung disorders, serositis, pulmonary vasculitis, necrobiotic nodules, drug-induced lung disease, thromboembolic lung disease and enteropulmonary fistulas. Pulmonary involvement may often be asymptomatic and detected solely on the basis of abnormal screening tests. The common embryonic origin of the intestine and the lungs from the primitive foregut, the co-existence of mucosa associated lymphoid tissue in both organs, autoimmunity, smoking and bacterial translocation from the colon to the lungs may all be involved in the pathogenesis of PM in IBD. PM are mainly detected by pulmonary function tests and high-resolution computed tomography. This review will focus on the involvement of the airways in the context of IBD, especially stenoses of the large airways, tracheobronchitis, bronchiectasis, bronchitis, mucoid impaction, bronchial granulomas, bronchiolitis, bronchiolitis obliterans syndrome and the co-existence of IBD with asthma, chronic obstructive pulmonary disease, sarcoidosis and a1-antitrypsin deficiency.

Key words: Inflammatory bowel diseases; Airways; Bronchiolitis

Core tip: The lung is commonly involved in inflammatory bowel diseases; however, airway involvement is often overlooked. This review will help gastroenterologists recognize the involvement of the airways in the context of inflammatory bowel diseases (IBD), especially stenoses of the large airways, tracheobronchitis, bronchiectasis, bronchitis, mucoid impaction, bronchial granulomas, bronchiolitis, bronchiolitis obliterans syndrome and the co-existence of IBD with asthma, chronic obstructive pulmonary disease, sarcoidosis and a1-antitrypsin deficiency, and appropriately manage their patients.

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INTRODUCTION

Extraintestinal manifestations (EIM) commonly occur in inflammatory bowel diseases (IBD), with a prevalence rate between 21%-41% reported in various series. Crohn's disease (CD) presents with EIM more frequently than ulcerative colitis (UC)[1]. The most common EIM are erythema nodosum, pyoderma gangrenosum, arthritis, uveitis,

episcleritis, mouth ulcers, renal stones, thromboembolic disease and primary sclerosing cholangitis. Pulmonary involvement complicating IBD was originally considered rare (with a frequency rate < 1%), but the first case series published in 1976 assisted in better recognition, evaluation and description of IBD related respiratory disease[2].

Pulmonary manifestations (PM) of IBD have been studied in the literature by small in size case-control studies, case reports and epidemiological population-based studies. Depending on the anatomic site involved, PM in IBD may be divided in to airway disorders, interstitial lung disorders, serositis, pulmonary vasculitis, necrobiotic nodules, drug-induced lung disease, thromboembolic lung disease and enteropulmonary fistulas. Concomitant occurrence of IBD with specific respiratory diseases [granulomatosis with polyangiitis (GPA), asthma, chronic obstructive pulmonary disease (COPD), alpha 1 antitrypsin deficiency and sarcoidosis] is not uncommon. Pulmonary involvement is often asymptomatic and may be detected solely on the basis of abnormal screening tests. This review will focus on the involvement of the airways in the context of IBD.

EPIDEMIOLOGY

The exact incidence and prevalence of PM in IBD is not known; however, airway involvement constitutes a large proportion, responsible for 40%-63% of overall respiratory incidents[3,4]. PM in total and specifically airway involvement seem to occur more commonly in UC than in CD.

Although IBD related PM were originally considered rare, certain population-based studies have revealed significant interrelationships between the lungs and IBD. Bernstein and colleagues in a large population-based study in North America in 2005 reported that airway disorders in general (including asthma and bronchitis) were the most common extraintestinal manifestation in subjects with CD and the second most common in subjects with UC, with the prevalence of asthma in this population between 7%-8%[5]. In another retrospective study from the opposite view, Birring reported that IBD was 4 times more prevalent among patients with airway diseases, particularly non-asthmatic patients with productive cough, than in the general population[6].

These epidemiological studies along with the observation of concordant EIM among siblings and first degree relatives suffering from IBD led to genome-wide studies that showed certain genetic predispositions for various EIM. Hence, in CD, HLA-A2 and HLA-DR1 and in UC, haplotypes HLA-B27, HLA-B58 and HLA-B8/DR3 are all linked to IBD related skin, joint and eye disorders[7,8]. To our knowledge, such a genetic predisposition for respiratory involvement in IBD has not been demonstrated yet.

PATHOGENESIS

Current theory for the pathogenesis of IBD postulates that in genetically susceptible individuals, environmental triggering factors cause a local immunologically aberrant intestinal injury and repair as a response to commensal bacteria. Environmental factors implicated are smoking (for CD), stress, infection, drugs and diet. Polymorphisms in NOD2-CARD15 (caspase recruitment domain family member 15) are found in 25%-35% of patients of European descent with CD; haplotype HLA DRB1*0103 within major histocompatibility complex (MHC) is associated with susceptibility to and extensive UC. Various genetic mutations (ATG16L1, IL-23 receptor, TNF polymorphisms) confer to result in disease progression by loosening of the intestinal epithelial barrier, decreasing of microbial clearance, loss of tolerance of the mucosa to enteric microflora (*autophagy*) and immunological dysregulation. Innate and adaptive immunity recognize microbial antigens and through pattern recognition receptors [toll-like receptors (TLR), nucleotide binding domain like receptors (NLR)] initiate an abnormal T-cell expansion, in the form of Th-1 and Th-17 pathways in CD and mainly Th-2 pathway in UC, that lead to chronic mucosal inflammation and disease[9,10].

Although significant progression is noted in the understanding of the pathogenesis of IBD, the exact mechanisms responsible for the cross-talk between bowel disease and airway disorders are not clearly elucidated. This association is important since clinical observations have repeatedly pointed out the phenomenon of a respiratory exacerbation occurring suddenly in patients with IBD after a therapeutic intervention (enterectomy) for their colon disease, with respiratory disease being completely unresponsive to this intervention[11]. However, the common embryonic origin of the intestine and the lungs from the primitive foregut should be considered the basis for this association. Their common origin reflects their common structural features: an extensive luminal surface area, protected by a tight epithelial barrier that covers a submucosal layer of goblet cells, glands and, most importantly, lymphoid tissue responsible for homing of lymphocytes, as well as innate and adaptive immunity[12].

Bronchus associated lymphoid tissue (BALT) and gut associated lymphoid tissue (GALT) are both parts of the mucosa associated lymphoid tissue (MALT). Lymphocytes become activated according to the inflammatory stimuli they receive and mis-homing of lymphocytes may provide an explanation for the migration of inflammation[13,14]. Furthermore, as we described earlier, apart from immune-mediated phenomena, autoimmunity [pANCA, anti-Saccharomyces cerevisiae antibodies (ASCA) and antibodies against intestinal epithelial antigens] also contributes to the pathogenesis of IBD[15]. It is therefore likely that based on common structures and the affinities of the lymphoid tissue, circulating immune complexes, stimuli and autoantibodies migrate from the intestine to bronchial and possibly alveolar epithelium, leading to airway inflammation and disease. This shift of inflammation may become more dramatic when the colon is removed after colectomy. In line with this, Adenis and colleagues in a scintigraphy study demonstrated increased pulmo-

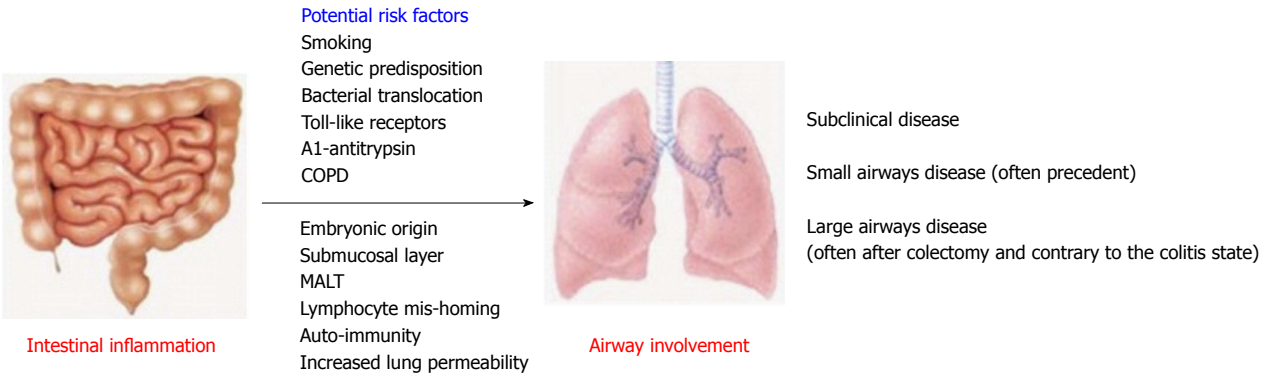

Figure 1 Possible pathogenesis of airway involvement and disease in the event of inflammatory bowel diseases. COPD: Chronic obstructive pulmonary disease; MALT: Mucosa associated lymphoid tissue.

nary permeability in patients with CD[16].

Certain contributing factors to such presumed pathogenesis may be proposed. Smoking is a well-known risk factor both for airway diseases and CD[17]. Bacterial translocation occurring in IBD may well affect the lung microbiome and confer to result in airway disorders since we already know that abnormal microbiome of the lungs carries implications in the pathogenesis of COPD[18]. Common TLR molecules (TLRs 2 and 4) participate in the pathogenesis of both COPD and IBD[19,20]. Lastly, matrix metalloproteinases (MMP) and anti-proteases like alpha-1 antitrypsin, well known as a cause of pulmonary emphysema, have been increasingly studied in the pathogenesis of IBD where their expression and balance seems to be disrupted, offering another potential link between the two systems[21]. A proposed schema for the pathogenesis of IBD related airway diseases is shown in Figure 1.

PATHOLOGY

As described earlier in this article, in the context of IBD, pathology examination may reveal abnormalities in different lung compartments, namely the airways, the interstitial tissue, the pleura, the parenchyma and the vessels. Interstitial pneumonitis and drug related eosinophilic pneumonia have been described; however, most intriguing for the pathologist is the differentiation between CD related pulmonary involvement and GPA in the case of pulmonary nodules, particularly since CD and GPA may coincide, as shown in several reports[22]. In this review, we shall focus on subclinical disease and airway pathology findings.

Bronchoalveolar lavage (BAL) studies have shown that chronic inflammation is common in the bronchi and alveoli of patients with CD. Wallaert *et al*[23] reported that 61% of asymptomatic patients with CD exhibit BAL features of an overt lymphocytic alveolitis. The clinical significance of this phenomenon, which provides further evidence for a systemic immunological manifestation of IBD to the lungs, is unknown. In our opinion, this alveolitis will not necessarily progress to clinical stage disease.

Small case series have described all types of biopsy proven bronchiolitis, documented with wedge and transbronchial biopsies mostly but also with open lung biopsies. Granulomatous bronchiolitis is more common; acute bronchiolitis with peribronchiolar inflammation, concentric small airways fibrosis, constrictive bronchiolitis and diffuse panbronchiolitis are also reported[4]. It should be highlighted that in the larger series by Casey and colleagues, bronchiolitides usually present before or concurrently with bowel disease, unlike other respiratory disorders that commonly follow bowel disease by a considerable time[24].

SCREENING FOR AIRWAY DISEASES

Airway disease, either latent and subclinical or clinically active, should be recognized for several reasons: (1) airway disease may complicate and follow a course independent of the course of the primary IBD; (2) IBD related airway diseases necessitate appropriate treatment and follow-up; (3) certain pulmonary function tests (PFT) may have a role as potential markers of disease activity; and (4) screening for respiratory disease may add to the recognition of concurrent diseases such as asthma and sarcoidosis. Screening for airway disease in the context of IBD may include medical history and clinical examination, PFT and radiological examinations.

Symptoms - clinical examination

Patients examined in an IBD clinic should be regularly asked about respiratory symptoms as nearly half of them report at least one symptom which they may attribute to anything but their primary disease. The most common symptoms reported are cough, sputum production, breathlessness mainly while exercising and wheezing. Stridor and hoarseness may develop in cases of upper airway narrowing.

Camus and colleagues reported that respiratory symptoms follow IBD presentation by months or even decades in nearly 85% of patients. In 10%-15%, respiratory symptoms precede and rarely (5%-10%) coincide with inflammatory bowel disease[3]. Respiratory symptom prevalence ranges from 25.6% in a recent study of 30 UC and 9 CD patients to 50% in an older study of 11

CD and 19 UC patients[25,26]. In a case control study of 64 IBD patients compared to 1346 controls, after adjustment for age, sex and smoking status, IBD patients were more likely to report shortness of breath and sputum production and to a lesser degree, cough [odds ratio (OR) respectively 3.4, 2.5, 1.8], highlighting the importance of relative clinical awareness[27].

Moreover, Higenbottam *et al*[28] described a respiratory exacerbation with cough and shortness of breath several years after coloproctectomy with the primary IBD in remission. This finding has been consistently described by other authors and several case reports. Thus, respiratory symptoms may occur independently of the course and activity of the bowel disease and are not responsive in parallel to its surgical treatment; on the contrary, pulmonary disease may be exacerbated. Most of these cases are reported with ulcerative colitis.

When respiratory symptoms occur acutely, the differential diagnosis includes pulmonary embolism, infectious pneumonia and drug toxicity. When respiratory disease follows an indolent course, airway disorders are more likely. Prompt evaluation in the latter scenario should include detailed medical history (with an emphasis on smoking habit, history of asthma and medication used), pulmonary function tests and radiological exams, including high resolution computed tomography scan (HRCT) of the chest with expiratory maneuvers. Bronchoscopy, as we will discuss further, is mandatory when there is evidence of upper airway involvement.

PFT

Symptomatic IBD patients may have normal PFT; this is apparently due to the anatomic site and the recruiting capacity of the airway system (bigger for small airways, smaller for large airways). On the other hand, up to two thirds of asymptomatic IBD patients have been found to demonstrate PFT abnormalities in recent studies, a surprising finding in light of the past reports of infrequent pulmonary involvement in IBD.

A prospective study of 40 IBD patients reported a 55% frequency of abnormal PFT in active IBD, equally distributed between UC and CD. This finding fell to 17.5% when IBD went into remission[29]. Herrlinger *et al*[30] reported 39% frequency of abnormal PFT in CD patients and 45% in UC, all asymptomatic, with values more affected during IBD activity but persisting in remission. Yilmaz *et al*[25] found abnormal PFT in 56% of IBD patients, with results directly affected by disease activity in UC cases. Another case-control study of 23 UC and 13 CD patients demonstrated abnormal PFT in 58% [75% of total events included low diffusing capacity for carbon monoxide (DLCO)], 81% of these patients with active IBD[31]. Lastly, in the larger study of 83 UC and 12 CD patients, Desai *et al*[32] reported abnormal PFT in 28.5% and low DLCO in 18%. Most of these studies included a mixed CD and UC population. UC patients, however, usually outnumbered CD patients, perhaps making PFT abnormalities seem more frequent in UC.

Decreased forced expiratory volume in the 1st second (FEV1), decreased FEV1/forced vital capacity (FVC) ratio, increased residual volume (RV)/total lung capacity (TLC) ratio, low forced expiratory flow (FEF_{25-75}) and, more importantly, decreased DLCO are the parameters noted to be abnormal in IBD patients in the existing literature. While FEV1 and FEV1/FVC ratio have been found to be normal in certain older studies, other studies and recent data suggest mild functional compromise[33,34]. Results should be interpreted with caution since different criteria have been used to define abnormal and certain studies included smoking patients or an inappropriate control population. In current studies, when an obstructive pattern has been noted, it demonstrates only partial reversibility which helps to differentiate it from asthma. RV/TLC ratio elevation has been demonstrated to correlate with bowel disease activity in several studies[35]. Tzanakis *et al*[36] have thoroughly described small airways disease, particularly in the event of active UC or CD.

The most consistent finding is a reduction in DLCO that commonly correlates with disease activity. In a large study of 47 CD and 85 UC patients, decreased DLCO was found in 19% and 17.6% respectively, with values being worse when the primary disease was active[37]. In another major study, DLCO was abnormal in 53% of inactive UC and in 81% of active UC patients; DLCO also correlated with pathological intestinal disease activity grading[38]. In children with IBD where PFT abnormalities are rather rare, reduced DLCO was the only finding in 53% of 26 children with active CD[39]. In conclusion, PFT and DLCO abnormalities in asymptomatic IBD are frequent but particularly so in active UC. It is believed that, as a systemic inflammatory disease, IBD affects the lung, creating a mild pulmonary inflammation corresponding to bowel inflammation. It is currently unknown, however, whether DLCO could serve as a disease activity marker.

Bronchial hyperresponsiveness (BHR) seems to occur more frequently in IBD than in a control population. This was demonstrated in 71% of children with CD and in 41% non-atopic IBD patients[40,41]. In line with this, airway eosinophilia and deranged induced sputum have also been reported in IBD[42]. These features may be associated with concomitant asthma or be subclinical and attributed solely to the underlying IBD. Thus, the etiology of BHR IBD may be two-fold: atopic and secondary to intestinal mucosal inflammation via the increased lung permeability observed in IBD[16].

It is currently unclear if asymptomatic patients with PFT abnormalities will progress to clinical respiratory disease and if so what defines this progression. Until more knowledge is acquired, PFT abnormalities should dictate closer follow-up of these patients.

Radiology - high resolution computed tomography

Studies show that HRCT in patients with IBD often demonstrates abnormal findings. The most common findings include bronchial wall thickening and bronchi-

Table 1 Classification of airway diseases secondary to inflammatory bowel diseases[1,3,4]

Site of involvement	Manifestations	Percent of total PM
Upper extrathoracic and intrathoracic airways (larynx/glottis, trachea, mainstem bronchi)	Stenoses, tracheobronchitis, acute respiratory failure	7%-8%
Large airways	Bronchiectasis	23%-26%
	Simple chronic bronchitis without suppuration	10%-20%
	Mucoid impaction	
	Bronchial granulomas	
	Suppurative bronchitis	3%-8%
Small airways	Granulomatous bronchiolitis	3%-10%
	Acute bronchiolitis	
	Diffuse panbronchiolitis	
	Bronchiolitis obliterans syndrome	
Concomitant diseases involving the airways	Asthma	
	Chronic obstructive pulmonary disease	
	Sarcoidosis	
	A1 antitrypsin deficiency	

PM: Pulmonary manifestations.

ectasis, cysts, emphysema, ground glass opacities and reticulonodular opacities. Centrilobular nodules, "tree-in-bud" opacities, air trapping and a mosaic pattern in expiratory scans all constitute a bronchiolitis radiological appearance. The prevalence ranges from 22% to 89% and radiological findings may be independent of symptoms, PFT results and primary disease intestinal activity[32]. This is because these findings may not be solely attributed to primary IBD related pulmonary disease. Alternative diagnoses include COPD, smoking related bronchiolitis, smoking related interstitial lung abnormalities, drug toxicities, thromboembolic disease and infections.

AIRWAY DISEASES IN IBD

Airway inflammation and disease are the most prevalent and distinctive pattern of respiratory involvement in IBD and accounts for 40%-63% of the total of clinically significant pulmonary complaints. Airway diseases (AD) usually follow IBD presentation by many years or even decades and the opposite presentation is the exception; when AD occur, IBD is rather inactive. If left untreated, AD can lead to irreversible stenoses in the airway. The clinical manifestations depend on the exact anatomic site involved. A classification for AD is shown in Table 1.

Upper airways

Upper airway disease (UAD) in IBD is a rare entity that may involve pharynx, larynx, trachea and mainstem bronchi. A total of 24 cases have been reported in the literature. Exudative lesions affect the bronchial mucosa and may cause subglottic stenosis and tracheobronchitis. UAD has been described in both UC and CD, with UC being predominant. Usually, it occurs years after diagnosis of the bowel disease, with IBD stable or in remission. UAD can occur after colectomy, with the time interval being as short as 30 d.

UAD may present with hoarseness, stridor and severe respiratory distress or just with cough, phlegm and shortness of breath[43,44]. Physical examination may reveal wheezing during inspiration, expiration or both. Because the clinical presentation may mimic infection or asthma, a certain degree of clinical suspicion is required to suspect an insult to the upper airway as a consequence of IBD. PFT and radiology are helpful in the diagnosis. A flow-volume loop demonstrates variable extrathoracic obstruction or fixed upper airway obstruction with plateau at both phases of respiration. While chest radiography has subtle findings, a CT scan of the chest may show circumferential or nodular narrowing of the trachea and bronchi. Bronchoscopy is the diagnostic procedure of choice[45]. Mucosal inflammation with exuberant pseudotumoral lesions, deformities, whitish lesions and narrowing of the lumen have all been described. Histology shows neutrophilic inflammation, granulation tissue, ulcerations, squamous cell metaplasia and plasma cell submucosal infiltrates. Noncaseating bronchial granulomas have also been reported in CD.

The differential diagnosis of IBD related UAD includes sarcoidosis, tuberculosis, amyloidosis and GPA[46]. The clinical presentation may be confusing if there is no bowel disease activity or other extraintestinal manifestations of IBD or if there is anti-neutrophil cytoplasmic antibodies (ANCA) positivity. ANCA are found to be positive in 50%-90% of UC and 10%-20% of CD patients but usually are neither cytoplasmic nor perinuclear in location[47]. The ANCA type specificity and histology of the airway lesions may help differentiate IBD-related UAD from GPA. Interestingly, isolated ANCA positivity without vasculitis has been associated with isolated subglottic stenosis in one study[48].

Empirical data suggest initial treatment with systemically administered high doses of corticosteroids (prednisone 1 mg/kg of lean body weight administered orally or methylprednisolone 60-80 mg intravenously per day); these suggestions are drawn from case reports, however, and not from randomized controlled trials, and should therefore be critically viewed[3]. In refractory cases, rigid bronchoscopy and interventional bronchoscopy procedures (laser beam, balloon dilation, stent placement) may be required in order to maintain an adequate airway[49,50].

Large airways

Large airways include the bronchi from the level of lobar bronchi to the level of terminal bronchioles. Large airways are the most common anatomic site of respiratory involvement in IBD, accounting for about 50% of total PM. Bronchial disease is more common in non-smoking females in their 5th decade of life in UC, particularly when other extraintestinal manifestations are present. Bronchial disease occurs many years after IBD in 8%-85% of pa-

tients, precedes IBD in younger patients (10%-15%) and less often coincides with active IBD (5%-10%). In 79% of cases, IBD is inactive and 50% of reported bronchial disease has followed colectomy[3,4].

The main large airway disorders are bronchiectasis (BE), chronic bronchitis (CB), suppurative bronchitis and mucoid impaction (Table 1). The classification depends on the patient's symptoms (the presence or absence of copious purulent sputum in the absence of BE classifies patient as suppurative bronchitis or CB, respectively) and HRCT features. Such features include luminal dilatation with bronchial wall thickening (definition of BE), bronchial wall thickening alone (CB) and mucoid impaction.

PFT usually reveal an obstructive pattern non-reversible to bronchodilators, or occasionally a mixed obstructive and restrictive pattern. CB diagnosis may be difficult to establish, particularly in long standing respiratory illnesses or in the presence of smoking. However, it should be noted that in most studies the reported patients were never smokers. Moreover, UC is epidemiologically connected to non-smoking individuals; in this setting, diagnosis of CB becomes more straightforward.

BE is the most important IBD related lung disorder. Typically, UC patients appear to present more commonly than CD. The bowel disease is often inactive and patients present with subacute symptoms of sputum production, cough and shortness of breath. In half of IBD-BE cases, a curative surgery (colectomy) has preceded the diagnosis of BE. In this setting, a close temporal relationship of weeks to months is well documented[11]. Spira and colleagues reported 6 UC and 1 CD patient with BE and CB in half of them manifesting after colectomy[51]. In another study of 14 UC and 3 CD patients, 76% developed BE, 41% shortly after enterectomy; such features are verified by other studies as well[3,52]. The most popular explanation for this sequence implicates a shift of mediators from the resected bowel to the lung, based on their common embryonic origin. In addition, withdrawal of immune-modulatory medication like corticosteroids after IBD remission may play a role in the flare up of pulmonary disease. Autoimmunity, as postulated by other authors, may also play a role as antinuclear antibodies have been discovered in some cases[53].

Importantly, bronchial disease is treated separately and independently from the bowel disease. As such, colectomy would aggravate rather than palliate bronchial disease. Treatment is the same as with any other cause of non-cystic fibrosis bronchiectasis. Antibiotics, bronchial toilet and bronchodilators should be offered as usual in all BE. Historically, certain authors advocate the use of corticosteroids in IBD-BE, based on case series and personal experience. These authors suggest inhaled corticosteroids should be used initially and if response is poor suggest prednisone administered orally at a dose of 0.5 mg/kg. Methylprednisolone has been also lavaged through the bronchoscope directly into the airways. Since no hard evidence supports the use of corticosteroids in IBD-BE or in bronchiectasis in general, and because of the concern for long term treatment with corticosteroids (CS), it is the authors' opinion that CS should not be administered in primary IBD related airway diseases[3,46,54].

Small airways

Small airways refer to the transitional airway zone from terminal bronchioles to alveolar ducts. Although larger case series attribute bronchiolitides as only 3%-10% of total IBD related pulmonary manifestations, their true involvement may be greater[4]. Kelly and colleagues evaluated 10 patients with IBD and bronchiectasis and found that 70% of them had abnormal FEF_{25-75}, suggesting that subclinical small airways disease is more frequent[55].

Bronchiolitides, the main form of small airway disorders, share a different clinical presentation than the clinical characteristics of upper and large airways involvement. They occur at a younger age, earlier in the disease course and in 1/3 of cases, pulmonary disease precedes intestinal disease[4]. As such, patients with bronchiolitis who have yet to be diagnosed with IBD often undergo invasive diagnostic procedures (bronchoscopy, open lung biopsy). In contrast to other pulmonary manifestations of IBD, the bronchiolitides are equally relevant in Crohn's disease and ulcerative colitis, as opposed to rest of airway disorders that are more prevalent in UC.

Pulmonary pathology in small airway involvement has been described earlier in this article. Granulomatous bronchiolitis is the most common finding, accounting for 59% of cases, and relates to CD as a systemic granulomatous disease[24]. PFT commonly demonstrates an obstructive pattern that may derange FEV1 or only FEF_{25-75}. DLCO is often abnormal as well[36]. HRCT features are the same as for all bronchiolitides (as already described).

This secondary bronchiolitis may be acute or, more commonly, chronic. Chronic bronchiolitis that persists untreated significantly worsens prognosis since it may eventually progress to diffuse airway narrowing and bronchiolitis obliterans syndrome (BOS). It may also lead to the progressive formation of bronchiolectasis and bronchiectasis[56]. This development is important because it explains physiologically the coexistence of small airways disease with larger airway involvement observed in patients with IBD. Since CS have a modest effect on small airways disease, we suggest the use of macrolides in the setting of IBD related bronchiolitis. Macrolides have shown clinical benefit in diffuse panbronchiolitis and BOS; azithromycin is shown to inhibit epithelial to mesenchymal transition and fibrosis to the small airways[57]. Nevertheless, in cases of BOS and despite therapy, transplantation may eventually be needed.

Concomitant diseases involving the airways

Asthma: After arthritis, asthma is the most common comorbidity in both UC and CD. A large population-based epidemiological study at the University of Manitoba compared 3873 UC cases with 38674 matched controls and found a 7.88% incidence of asthma in UC (especially in males); similarly, they studied 4187 CD cases with

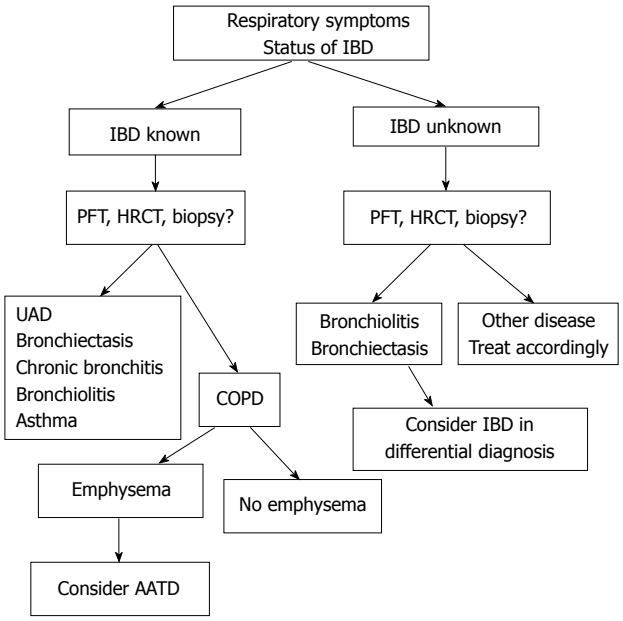

Figure 2 Proposed diagnostic algorithm for the evaluation of airway disease in inflammatory bowel diseases. IBD: Inflammatory bowel disease; PFT: Pulmonary function tests; HRCT: High resolution computed tomography; UAD: Upper airway disease; COPD: Chronic obstructive pulmonary disease; AATD: A1 antitrypsin deficiency.

41815 matched controls and found a 7.09% prevalence ratio of asthma in CD (especially in females)[5].

We have already discussed the increased prevalence of BHR and atopy in IBD. It should be noted that when a clinical phenotype of asthma is established in a patient with IBD, appropriate treatment is mandatory since there is epidemiological evidence of increased mortality in the asthma-UC population and laboratory evidence of more severe BHR in asthma-UC patients[58,59].

COPD: Cigarette smoke is known to protect against UC but promotes CD progression. Another population-based study investigated the relationship between COPD and IBD. Investigators found that COPD cases had a 1.83 hazard ratio (HR) for UC and a 2.72 HR for CD; this hazard extended to first degree relatives. As such, an inflammatory vulnerability in COPD patients has been postulated[60]. A very recent study evaluating the intestinal function of COPD patients demonstrated that COPD, regarded as a systemic inflammatory disease, causes intestinal hyperpermeability and enterocyte damage leading to intestinal compromise. The latter potentially provides an explanation for this coincidence from an etiological and environmental point of view[61].

COPD in IBD patients should be investigated, recognized and treated appropriately. COPD increases all-cause mortality and specific cause mortality in patients with CD. A meta-analysis by Duricova and colleagues reported a standardized mortality rate of 2.55 for CD-COPD subjects[62].

Sarcoidosis: Sarcoidosis shares many common characteristics with IBD, especially CD, as they both are granulomatous diseases. Sarcoidosis and IBD may coincide, as shown by case reports and population-based studies. They both share multi-organ manifestations (joints, eye, skin). Sarcoidosis and IBD seem to share a genetic overlap regarding cytoplasmic nucleotide oligomerization domains 1 and 2 and certain polymorphisms in chromosomes 1 (loci *IL-23R* and *1q.24.3*) and 15 (locus *HERC2*)[63].

IBD, as discussed earlier in this article, may exhibit granulomatous lung disease, mainly bronchiolitis. If infections have been ruled out, it is intriguing to differentiate between an IBD pulmonary localization and concomitant sarcoidosis. Histopathological features pointing to sarcoidosis are a lymphangitic distribution of granulomas and the absence of interstitial pneumonia and chronic bronchiolitis[24]. A clinical approach, however, is mandatory as the diagnosis of sarcoidosis apart from granuloma pathology demands compatible clinical and radiological findings.

A1 antitrypsin deficiency: Heterozygosity for the PiZ allele of a1 antitrypsin (AAT) deficiency (AATD) has been found to be more prevalent in patients with UC than in the general Swedish population (8.5% *vs* 4.7%)[64]. More importantly, a recent study from the United Kingdom confirmed a higher prevalence of UC among subjects suffering from emphysema due to homozygous PiZZ allele and AATD in comparison to the general population (1.5% *vs* 0.4%)[65]. Consequently, a blood test for AATD should be ordered when emphysema and IBD coincide in a young patient. It is unknown if AAT supplementation could be of use in the therapy of IBD.

Table 2 Key messages

In a patient with IBD and respiratory symptoms, symptoms should be initially attributed to the primary disease because of significant lung-intestine interference
IBD, asthma and COPD often coincide
IBD should be always remembered in the differential diagnosis of bronchiectasis and bronchiolitis
PFT and HRCT are necessary to evaluate a symptomatic patient
IBD related airway disease does not necessarily follow the course of colitis

IBD: Inflammatory bowel disease; COPD: Chronic obstructive pulmonary disease; PFT: Pulmonary function tests; HRCT: High resolution computed tomography.

CONCLUSION

Patients with IBD may present sometime during the course of their disease with various pulmonary incidents. The clinical approach relies upon the doctor's knowledge and judgment to attribute the patient's symptoms to the primary disease, comorbidities or to other complications after a thorough investigation. A proposed diagnostic algorithm for the evaluation of respiratory disease in IBD is shown in Figure 2. Table 2 contains the key messages

that, in our opinion, summarize the pulmonary-intestinal interrelationships in inflammatory bowel diseases.

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MINIREVIEWS

Genetic and environmental determinants of risk for cholangiocarcinoma in Thailand

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Abstract

Cholangiocarcinoma (CCA) is a difficult cancer to diagnose in the early stage and to treat by curative resection. The incidence of CCA in the northeast of Thailand is the highest in the world. To make progress in detecting a high risk group and in the prevention and detection of CCA, we have been analyzing the risk factors for CCA. Although liver fluke infection is known to be a risk factor, there are patients who are not infected with the liver fluke and not all people infected with the liver fluke will suffer from the disease. Therefore, it is of the utmost importance to analyze the risk factors and the mechanism to prevent the disease and also to detect the disease in its early stage to save patients' lives. Through collaboration among Thai and Japanese researchers, we analyzed the genetic and environmental determinants of risks for CCA. Also, we have been trying to develop methods to detect the disease in a non-invasive way. Without repeating findings reported in various reviews on CCA, we will first discuss the environmental and genetic determinants of the risks for CCA. Second, we will discuss the properties of CCA, including the etiological agents and the mechanism of cholangiocarcinogenesis, and finally, we will discuss future approaches to prevent and cure CCA from the standpoint of evidence-based medicine. We will discuss these points by including the data from our laboratories. We would like to emphasize the importance of the genetic data, especially whole genome approaches, to understand the properties of CCA, to find a high risk population for CCA and to develop effective preventative methods to stop the carcinogenic steps toward CCA in the near future. In addition, it is of the upmost importance to develop a non-invasive, specific and sensitive method to detect CCA in its early stage for the application of modern medical approaches to help patients with CCA.

Key words: Alcohol drinking; Cholangiocarcinoma; DNA polymorphism; Glutathione S transferase; 8-oxoguanine

glycosylase 1; Liver fluke; *Opisthorchis viverrini*; Thailand

Core tip: Cholangiocarcinoma (CCA) is an intractable cancer due to the difficulty of diagnosis in its early stage. The incidence of CCA in the northeast of Thailand is the highest in the world. It is of the utmost importance to analyze the risk factors and the mechanism to prevent the disease and to also detect the disease in its early stage to save patients' lives. We analyzed the genetic and environmental determinants of risks for CCA and discussed this with the findings already published by other researchers. It is of the utmost importance to develop a non-invasive, specific and sensitive method to detect CCA.

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INTRODUCTION

The age standardized rates (world ASR) of the incidence of liver and bile duct cancer in Thailand between 2001 and 2003 are 38.6 and 14.6 for men and women respectively. Most remarkably, world ASR of liver and bile duct cancer in Udon Thani, Khon Kaen, Nakorn Phanom, Ubon Ratchathani, Bangkok and Songkhla provinces for men are 115.0, 87.7, 78.4, 74.9, 21.5 and 10.9, respectively, and for women are 52.7, 36.3, 43.2, 34.7, 6.4 and 2.9, respectively. Cholangiocarcinoma (CCA) among the liver and bile duct cancer in the above provinces for men are 80.5%, 81.1%, 55.9%, 81.0%, 32.6% and 33.3%, respectively, and for women are 86.7%, 82.3%, 60.8%, 76.6%, 56.7% and 43.5%, respectively[1]. Thus, the incidence of CCA in the northeast of Thailand is extremely high in comparison to the rest of the world.

It was previously reported that the liver fluke, *Opisthorchis viverrini* (OV), and endogenous nitrosamines are the important risk factors for CCA in Thailand[2,3]. Multiple pathways on the tumorigenic OV infection to cause CCA from Thailand are nicely summarized in the recent review[4].

ENVIRONMENTAL DETERMINANTS

From the epidemiological study, it was previously known that the infection of the liver fluke, OV, is an important risk factor of CCA[2] (Table 1). In addition to OV infection, some of the chemical carcinogens like nitrosamine are also suggested to be factors in the risk for CCA[3]. We performed a population-based case-control study in which sex, age and place of residence were matched individually. We confirmed that the presence of the antibody against OV significantly increased the risk for CCA; odds ratio (OR) = 27.09 [95% confidence interval (CI): 6.30-116.57]. The results confirmed the previously reported data by Parkin *et al*[2]. In addition, we found that alcohol drinking is another risk factor for CCA. Ex-regular and regular alcohol drinkers showed OR = 6.23 (95%CI: 1.23-31.57) and OR = 4.31 (95%CI: 1.12-16.57), respectively (Table 1)[5]. We examined the possibility that alcohol consumption affects the risk for CCA due to OV infection, as well as smoking and dietary habits during the past 10 years, and found only the risks due to smoking and eating fermented fish (*pla-ra* and/or *pla-chao*) were altered with alcohol consumption (P for interaction < 0.01 and 0.07, respectively). The interactions between alcohol drinking and selected variables are shown in Table 2. The odds ratios are slightly different from those appearing in our previous paper[5] due to a typing error although the conclusion is materially the same. The increased risk for CCA due to ever-smoking was more prominent among ever-drinkers than among never-drinkers and a similar observation was made for the risk by eating *pla-ra* and/or *pla-chao*. Conversely, vitamin C was suggested to reduce the risk[3]. Recently, Songserm *et al*[6] confirmed that alcohol drinking increased the risk for CCA and they reported that the consumption of fruits and vegetables decreased the risk for CCA (Table 1). Manwong *et al*[7] also reported that a family history of cancer was a significant risk factor (Table 1).

INTERACTION BETWEEN GENETIC AND ENVIRONMENTAL DETERMINANTS

Since not all patients with CCA are infected with OV and not all individuals infected by OV develop CCA, it is possible that some other environmental and genetic determinants are involved in the pathogenesis of CCA. We examined the genetic polymorphism on the risk for CCA. We first examined the effect of carcinogen detoxification enzyme gene polymorphisms, namely *GSTM1* and *GSTT1*, which are well-known. DNA polymorphism of *GSTM1* or *GSTT1* alone was not associated with the risk of CCA. However, the null genotype of *GSTM1* enhanced OR of the risk for CCA in anti-OV antibody positive subjects was 18.00 (95%CI: 3.33-97.40) compared to that of *GSTM1* wild in anti-OV antibody positive subjects of 10.34 (95%CI: 1.31-81.63) and the null genotype of *GSTT1* enhanced OR in ex-regular alcohol drinkers was OR = 27.93 (95%CI: 1.84-424.60) compared to that of *GSTT1* wild in ex-regular drinkers of OR = 1.28 (95%CI: 0.12-14.08)[5].

Songserm *et al*[6] analyzed methylenetetrahydrofolate reductase gene polymorphism (*MTHFR*) at 677 and at 1298 for interaction with beef sausage consumption (Table 3). They found that *MTHFR*677 TT variants and *MTHFR*1298 CC variants showed increased risks when the individuals ate beef sausage daily. The data attained by the above researchers which showed an interaction are listed in Table 3.

Table 1 Effects of environmental determinants on risks for cholangiocarcinoma

Environmental determinants		Cases	Controls	OR	95%CI		*P* value	Ref.	Ethnic group
					LL	UL			
Anti-OV Ab	ref: < 1/40	101 matched case-control pairs		5.00	2.30	11.00	< 0.001	Parkin *et al*[2] 1991	Thai
				Adjusted OR					
Anti-OV Ab (ELISA)	< 0.200	61	119	1.00	Reference			Honjo *et al*[5] 2005	Thai
	⩾ 0.200	65	8	27.09	6.30	116.57	< 0.01		
Alcohol drinking	Never	30	46	1.00	Reference		-		
	Occasional	41	54	2.20	0.65	7.45	0.21		
	Ex-regular	15	7	6.23	1.23	31.57	0.03		
	Regular	41	21	4.31	1.12	16.57	0.03		
	Missing	2	-	-	-	-	-		
Raw fish	0	30	57	1.00	Reference				
	< 2/mo	54	41	2.70	1.28	5.68	< 0.01		
	⩾ 2/mo	45	31	2.94	1.24	6.96	0.01		
Fermented fish or pork	0	28	41	1.00	Reference				
	< 2/mo	58	63	2.95	0.98	8.90	0.06		
	⩾ 2/mo	43	25	4.50	1.30	15.54	0.02		
				Adjusted OR					
Alcohol drinking	Non-drinker	57	254	1.00	Reference			Songserm *et al*[6] 2012	Thai
(Units of alcohol per month)	< 14	79	92	5.60	2.85	10.95	< 0.001		
	⩾ 14	83	92	9.50	4.55	19.79	< 0.001		
Total vegetables (average times/ month)	< 52	136	214	1.00	Reference				
	⩾ 52	83	224	0.40	0.23	0.76	0.004		
Total fruits (average times/ month)	< 35	131	217	1.00	Reference				
	⩾ 35	88	221	0.60	0.33	0.98	0.04		
Family history of cancer	No	85	107	1.00	Reference			Manwong *et al*[7] 2013	Thai
	Yes	38	16	4.34	1.80	10.43	0.001		

OR: Odds ratio; CI: Confidence interval; LL: Lower limit; UL: Upper limit.

EFFECTS OF GENETIC DETERMINANTS AND DNA POLYMORPHISM ON RISK FOR CCA

There are several reports of the effects of DNA polymorphisms on the risk of CCA. Among various enzymes involved in carcinogen metabolism, CYP1A2, one of the phase I enzymes in the activation of such a carcinogen in cigarette smoke, has a DNA polymorphism. *CYP1A2* polymorphism, found in intron 1, might be involved in the risk of CCA. Prawan *et al*[8] found that *CYP1A2*1A/*1A* polymorphism had a protective effect on the risk of CCA in men but not in women (Table 4). Since men smoke more than women in Thailand, it is considered that in the individuals with *CYP1A2*1A* polymorphism, the CYP1A2 enzyme might be less inducible compared to that with *CYP1A2*1F*, although the effect of these mutations on the induction of the enzyme is not clear.

Arylamine *N*-acetyltransferase (NAT) catalyzes *N*- and *O*-acetylation of various arylamines and heterocyclic amines, thereby regulating the metabolic activation and detoxification of xenobiotics and carcinogens. Individuals with three *NAT2* alleles, *NAT2*13*, **6B* and **7A*, are associated with a decreased risk for CCA, while those with *NAT2*4, *5, *6A* and **7B* were not, suggesting that the *NAT2* polymorphism may modify the risk of CCA (Table 4)[8].

Glutathione *S*-transferases (GSTs), a family of Phase II detoxifying enzymes, can conjugate reduced glutathione to various compounds. Concerning polymorphism of *GSTO1* and *GSTO2*, Marahatta *et al*[9] found that individuals with *GSTO1*D140* had a significantly increased risk for CCA, hepatocellular carcinoma and breast cancer (Table 4). A study with a larger sample size will better clarify the function of GSTO1.

Natural killer cell receptor G2D (NKG2D) haplotypes were found to be associated with the natural cytotoxic activity of individuals. NKG2D triggers cell-mediated cytotoxicity in natural killer cells. Various NKG2D haplotype alleles showed a significant difference between cases and controls[10]. Primary sclerosing cholangitis (PSC) is an inflammatory bowel disease suggested to be a predisposing disease to hepatobiliary malignancy.

Table 2 Effect of modification of alcohol drinking on relationships between smoking, eating fermented fish and risk for cholangiocarcinoma

Variable	Category	Alcohol drinking							
		Never drinkers				Ever[1] drinkers			
		Adjusted[2] OR	95%CI		*P* value	Adjusted[2] OR	95% CI		*P* value
			LL	UL			LL	UL	
Smoking	Never	1	Reference			4.25	1.02	17.63	0.05
	Occasional	4.36	0.4	47.49	0.23	1.07	0.06	20.66	0.96
	Ex-regular					9.09	1.27	65.18	0.03
	Regular	3.64	0.19	71.41	0.39	7.99	1.56	40.94	0.01
Pla-ra,	< 3/d	1	Reference			14.07	1.46	135.36	0.02
Pla-chao	≥ 3/d	12.34	1.22	124.75	0.03	20.88	2.27	192.06	< 0.01

[1]Including occasional, ex- and currently regular drinkers; [2]Adjusted for anti-OV Ab when calculating the OR of smoking, and adjusted for anti-OV Ab and smoking when calculating the OR of eating of fermented fish (pla-ra and/or pla-chao). Nakorn Phanom (Thailand): based on the conditional logistic regression model. CI: Confidence interval; LL: Lower limit; UL: Upper limit; OR: Odds ratio. Adapted from Honjo *et al*[5] 2005. Allowing for absence of control subject in the category for occasional smoking and absence of case subject in the category for ex-regular smoking among never drinkers, we combined these two categories and confirmed the conclusion in the table is the materially unchanged from that in the table in our previous paper (Honjo *et al*[5] 2005).

Table 3 Interaction between genetic and environmental determinants on risks for cholangiocarcinoma

Genetic determinants		Environmental determinants		OR	95%CI		*P* value	Ref.	Ethnic group
					LL	UL			
				Adjusted OR					
GSTMI	Wild	Anti-OV antibody	Negative	1.00	Reference			Honjo *et al*[5] 2005	Thai
	Wild		Positive	10.34	1.31	81.63	0.03		
	Null		Negative	0.48	0.21	1.11	0.09		
	Null		Positive	18.00	3.33	97.40	< 0.01		
GSTMI	Wild	Toilet	Inside the house	1.00	Reference				
	Wild		Outside or none	0.20	0.04	1.02	0.05		
	Null		Inside the house	0.22	0.06	0.88	0.03		
	Null		Outside or none	0.25	0.07	0.91	0.04		
GSTTI	Wild	Alcohol drinking	Never	1.00	Reference				
	Wild		Occasional	3.58	0.71	17.95	0.12		
	Wild		Ex-regular	1.28	0.12	14.08	0.84		
	Wild		Regular	4.69	0.93	23.51	0.06		
	Null		Never	0.75	0.23	2.43	0.63		
	Null		Occasional	1.12	0.22	5.80	0.89		
	Null		Ex-regular	27.93	1.84	424.60	0.02		
	Null		Regular	3.28	0.35	30.91	0.30		
				Crude OR					
MTHFR 677	CC	Beef sausage	< 1/mo	1.0	Reference			Songserm *et al*[6] 2012	Thai
	CT		< 1/mo	1.1	0.51	2.37	0.82		
	TT		< 1/mo	0.6	0.25	1.53	0.32		
	CC		Weekly	0.9	0.45	1.83	0.80		
	CT		Weekly	1.2	0.57	2.43	0.65		
	TT		Weekly	1.6	0.80	3.31	0.18		
	CC		Daily	3.3	1.51	7.07	0.003		
	CT		Daily	3.2	1.33	7.62	0.01		
	TT		Daily	8.3	2.23	30.82	0.002		
MTHFR 1298	AA	Beef sausage	< 1/mo	1.0	Reference				
	AC		< 1/mo	1.3	0.63	2.55	0.51		
	CC		< 1/mo	0.8	0.28	2.15	0.63		
	AA		Weekly	1.3	0.71	2.45	0.39		
	AC		Weekly	1.0	0.49	1.79	0.84		
	CC		Weekly	3.8	1.48	9.89	0.01		
	AA		Daily	3.8	1.71	8.62	0.001		
	AC		Daily	3.5	1.56	7.85	0.002		
	CC		Daily	18.3	3.68	90.80	< 0.001		

Thirteen percent of patients with primary sclerosing cholangitis developed CCA[11]. When NKG2D single nucleotide polymorphisms (SNPs) were compared between the PSC patients with CCA and the PSC patients

Table 4 Effects of genetic determinants on risks for cholangiocarcinoma

Genotype		No. CCA (%)	No. control (%)	OR	95%CI		*P* value	Ref.	Ethnic group
					LL	UL			
				Adjusted OR					
CYP1A2, Male	*1F/*1F	85 (57.4)	88 (51.2)	1.0	Reference			Prawan *et al*[8] 2005	Thai
	*1A/*1F	59 (39.9)	69 (40.1)	0.9	0.55	1.47	0.677		
	*1A/*1A	4 (2.7)	15 (8.7)	0.28	0.08	0.94	0.039		
NAT2	All, except *6B, *7A and *13	193 (89.4)	162 (69.5)	1.0	Reference				
	One or two alleles (All, except *6B, *7A and *13)	23 (10.6)	71 (30.5)	0.26	0.15	0.44	< 0.001		
				Crude OR					
GST01	A140/A140	13 (43.33)	26 (86.67)	1.0	Reference			Marahatta *et al*[9] 2006	Thai
	A140/D140 + D140/D140	17 (56.67)	4 (13.33)	0.86	2.07	37.85			
		Minor allele frequency							
	Alleles	PSC[2] with CCA (n = 49)	PSC without CCA (n = 316)	OR			Corrected P		
NKG2D[1]	rs11053781 (Intron 5) G *vs* A	0.66	0.49	2.08	1.31	3.29	0.011	Melum *et al*[12] 2008	Norwegian
	rs2617167 (Intron 1) A *vs* G	0.39	0.22	2.32	1.47	3.66	0.002		
		PSC with CCA (n = 49)	Healthy controls (n = 368)						
	rs11053781 (Intron 5) G *vs* A	0.66	0.5	1.95	1.23	3.07	0.021		
	rs2617167 (Intron 1) A *vs* G	0.39	0.23	2.2	1.40	3.44	0.003		
		Counts (frequencies) of alleles/genotypes							
		2n = 120	2n = 146	Crude OR				Hoeblinger *et al*[13] 2009	Caucasian
MRP2/ ABCC2[3]	*ABCC2 c.3972* C (exon 28, synonymous SNP)	73 (0.61)	108 (0.74)						
	ABCC2 c.3972 T	47 (0.39)	38 (0.26)	1.83	1.087	3.08	0.022		
				OR					
MYH rs3219476	T/T	25 (42.4)	26 (26.0)	1.0	Reference			You *et al*[14] 2013	Han Chinese
	T/G	20 (33.9)	58 (58.0)	0.359	0.17	0.758	0.006		
	G/G	14 (23.7)	16 (16.0)	0.91	0.369	2.246	0.838		
	T/G + G/G	34 (57.6)	74 (74.0)	0.478	0.241	0.946	0.033		
MYH rs3219472	G/G	28 (47.5)	46 (46.0)	1.0	Reference				
	G/A	19 (32.2)	47 (47.0)	0.664	0.326	1.351	0.258		
	A/A	12 (20.3)	7 (7.0)	2.816	0.992	7.999	0.047		
	G/A + A/A	31 (52.5)	54 (54.0)	0.943	0.495	1.797	0.859		

[1]Natural killer cell receptor G2D; [2]Primary sclerosing cholangitis; [3]Multidrug resistance-associated protein 2 gene. OR: Odds ratio.

without CCA in a Norwegian population, there was significantly increased allele frequencies in two SNPs, namely rs11053781 and rs11053781, both of which are non-coding. The odds ratio for G *vs* A in the rs11053781 was 2.08 (95%CI: 1.31-3.29) and that for A *vs* G in rs2617167 was 2.32 (95%CI: 1.47-3.66). When they were compared between PSC patients with CCA and healthy controls, there was also a significant increase of allele frequencies in the above two SNPs. The odds ratio for G *vs* A in the rs11053781 was 1.95 (95%CI: 1.23-3.07) and that for A *vs* G in rs2617167 was 2.20 (95%CI: 1.40-3.44) (Table 4)[12]. The functional role of the changes of these SNPs on the susceptibility to CCA remains to be elucidated.

Multidrug resistance-associated protein 2 (MRP2/ABCC2), one of the ATP-binding cassette transporter proteins, is suggested to be involved in the excretion of the conjugates of carcinogens into bile, a metabolic step classified as so called "Phase Ⅲ metabolism". Thus, it might play an important role in cellular defense against toxic substances. The frequency of the *c.3972C > T ABCC2* gene variant (synonymous SNP) was compared between patients with CCA and healthy individuals.

Table 5 Interaction among genetic determinants on risks for cholangiocarcinoma

Genetic determinant		Genetic determinant		OR	95%CI		*P* value	Ref.	Ethnic group
					LL	UL			
MTHFR C677T[1]	CC	*TSER*[2]	*2R* (-)	1	Reference			Ko *et al*[17] 2006	South Korean
	CC		*2R* (+)[3]	5.38	1.23	23.56	0.026		
	CT		*2R* (-)	1.08	0.68	1.07			
	CT		*2R* (+)[3]	1.19	0.71	2.01			
	TT		*2R* (-)	1.02	0.7	1.5			
	TT		*2R* (+)[3]	1.24	0.9	1.71			
hOGG1(*Codon326*)	Ser/Ser	*GSTM1*	wild	1	Reference			Zeng *et al*[18] 2013	Thai
	Ser/Ser + Cys/Cys		wild	0.06	0.01	0.54	0.01		
	Ser/Ser		null	0.06	0.01	0.53	0.01		
	Ser/Ser + Cys/Cys		null	0.14	0.02	1.08	0.06		

[1]5,10-Methylenetetrahydrofolate reductase; [2]Thymidylate synthase enhancer region; [3]Including 2R2R and 2R3R.

There was a significant association between the SNP and the risk in a Caucasian population (Table 4)[13].

The DNA repair mechanism is protecting DNA damage caused by various kinds of carcinogenic factors. Among them, base excision repair (BER) plays an important role in the oxidative DNA damage caused by reactive oxygen species. MutY homolog, MYH, is involved in BER and functions as a DNA glycosylase which removes adenine paired with 8-hydroxy-2'-deoxyguanine residue. Individuals with T/G genotype in MYH rs3219476 had a reduced risk (OR = 0.478, 95%CI: 0.17-0.758, $P = 0.006$). Individuals with A/A genotype in MYHrs3219472 had an increased risk (OR = 2.816, 95%CI: 0.992-7.999, $P = 0.047$) (Table 4)[14].

Concerning other variants or mutations related to the risk for CCA, a mutation in bile salt export pump (ABCB11) was found in two children with progressive familial intrahepatic cholestasis and cholangiocarcinoma[15]. Biliary papillomatosis is considered to be a premalignant lesion with a high probability to develop to CCA, although the genetic changes have not been clarified[16].

INTERACTION AMONG GENETIC DETERMINANTS

Susceptibility to cancer might be regulated not only by one gene or one environmental determinant. Thus, interaction of genetic determinants could easily be imagined in regulating various cellular processes. However, there are few reports on the interaction among genetic determinants. Ko *et al*[17] reported the interaction of polymorphisms of 5,10-methylenetetrahydrofolate reductase (MTHFR *C677T*) and thymidylate synthase enhancer region (TSER) and the risk for CCA in a South Korean population (Ko *et al*[17] 2006). MTHFR is involved in the pathway of folate metabolism and DNA methylation. Thymidylate synthase (TS) catalyzes the formation of dTMP from dUMP, an important step for production of dTTP for use in DNA synthesis. Both TS and MTHFR use the common substrate 5,10-methylanetetrahydrofolate and might affect DNA synthesis and repair. Therefore, the interaction between *MTHFR C677T* and *TSER* polymorphisms were analyzed. Ko *et al*[17] found that the individuals with *MTHFR 677CC* with *TSER 2R(+)* genotypes (2R2R, 2R3R, 2R5R) showed an increased risk for CCA compared to *677CC* with *TSER 2R(-)* genotypes (3R3R, 3R4R, 3R5R) ($P = 0.0257$) (Table 5)[17]. There was no association between *MTHFR C677T* polymorphism or *TSER* polymorphism alone and the risk for CCA.

Human 8-oxoguanine glycosylase 1 (hOGG1) is involved in the repair of 8-hydroxy-2'deoxyguanine residue in oxidatively damaged DNA, one of the most mutagenic lesions among base modification produced by reactive oxygen species. While polymorphisms of DNA repair enzymes, including hOGG1 (codon 326), XRCC1 (codon 194, 280 and 399) and PARP1 (codon 762), alone had no association with the risk for CCA[18], there is a significant interaction between hOGG1 and *GSTM1* polymorphisms for the risk for CCA. When *GSTM1* polymorphism was considered, the hOGG1 codon 326 polymorphism was related to the decreased risk: OR = 1.00 (reference), OR = 0.06 (95%CI: 0.01-0.53), OR = 0.06 (95%CI: 0.01-0.54) and OR = 0.14 (95%CI: 0.02-1.08) for subjects with hOGG1 Ser/Ser and *GSTM1* wild, ones with Ser/Ser and *GSTM1* null, ones with Ser/Cys or Cys/Cys and *GSTM1* wild, and ones with Ser/Cys or Cys/Cys and *GSTM1* null, respectively (P for interaction < 0.01) (Table 5). Although the effect of hOGG1 polymorphism is not clear when amino acid Ser 326 is changed to Cys, the DNA repair capacity might decrease. However, the above data showed the decreased risk of CCA. It could be considered that if DNA repair capacity is inhibited when relatively abundant DNA damage is present in the presence or absence of *GSTM1* enzyme, the cells would die before malignant transformation[18]. Kim *et al*[19] reported that hOGG1 326 Cys/Cys genotypes were associated with lowered risk of bladder cancer occurrence and recurrence in South Korean subjects, while hOGG1 326 Ser/Cys genotype was a risk factor. The protective effect of *GSTM1* null variant could be

due to the slow metabolism caused by *GSTM1* deficiency of some dietary materials, such as isothiocyanates contained in cruciferous vegetables, known to be a chemopreventive compound. The protective effects of *GSTM1* null variant were reported in breast carcinoma[20] and hepatocellular carcinoma[21]. The concerted action of a DNA-repair enzyme and *GSTM1* on the risk for CCA should give a new insight in understanding the mechanism of the carcinogenesis of CCA.

ETIOLOGICAL AND ENHANCING AGENTS FOR CHOLANGIOCARCINOGENESIS AND THEIR PATHOGENICITY

Concerning the etiological agents for CCA, epidemiological studies implicated various chemicals and occupational risks. One of the examples is thorium dioxide (thorotrast) used for radiological examination[22]. Animal experiments showed that *N*-nitrosodimethylamine could induce CCA in the Syrian Golden hamster[23]. Although OV infection alone did not induce CCA, the OV infection enhanced CCA production by *N*-nitrosodimethylamine in the hamsters[24,25]. Actually, a small amount of nitrosamine was detected in the food[4]. Quite recently, 1, 2-dichloropropane and/or dichloromethane used in the color proof-printing factory were considered to be the etiological agents from a precise epidemiological study in Japan[26]. Other than the liver fluke, viral infections like hepatitis B and C virus infections are also related to the increased risk for CCA[27].

There have been many findings on the abnormalities of gene expression caused by the reorganization of the genome through endogenous and environmental factors in many types of cancers[28]. It is also true for CCA that many genetic changes are found in CCA. One of the examples from our laboratory is the mutation of the tumor suppressor protein genes, *p16Ink4/CDKN2* and *p15Ink4B/MTS2*[29]. However, the precise mechanisms of cholangiocarcinogenesis are not well clarified. We have been using a hamster model of cholangiocarcinoma and found that a molecule, protein kinase A regulatory subunit 1 α (Prkar1a), is overexpressed in the cholangiocarcinoma tissues[30,31]. *PRKAR1A* gene overexpression is also found in humans and this is associated with production of extracellular protein kinase A (ECPKA), especially its catalytic subunit (PRKACA)[31], as found in prostate cancer. Although the function of the extracellular protein kinase A is not clear, it might contribute to the development of cancer cells[32].

The precise mechanism of liver fluke infection causing CCA (cholangiocarcinogenesis) is also not known. OV produces mechanical injury to the biliary epithelia by attachment with suckers, inflammation caused by OV and mitogenic factors secreted by OV to help the biliary epithelial cells transform to CCA[33]. In particular, TGF-β and EGF signal transduction pathways are indicated as the possible pathways of OV-induced cell proliferation of fibroblasts[34]. It could be speculated that CCA-associated fibroblasts induce tumor progression of the initiated epithelium, as found in human prostate epithelium[35]. This would be a novel target for chemoprevention and treatment of fibrosis in CCA which might delay the formation of CCA. Gene expression profile of OV infection-related CCA and non-OV associated CCA was reported by Jinawath *et al*[36]. Enhanced expression of RAD51 associating protein-1 was also involved in the growth of CCA cells[37]. These genes upregulated in CCA would be expected to serve as diagnostic and therapeutic targets for CCA. The up-to-date findings of the mechanism of tumorigenesis by OV infection and the prevention of OV infection, including the education and trial for vaccine development against OV, is reviewed by Sripa *et al*[4].

FUTURE PROSPECTS FOR PREVENTION AND EARLY DETECTION OF CCA

The present work is intended to analyze the effects of environmental and genetic determinants on the risk of CCA and to know the mechanisms of CCA to prevent the disease. At the same time, it is also important to detect the disease during its early phase so that medical intervention could possibly prevent the death of patients with cholangiocarcinoma. Therefore, the method to detect the high risk population and patients with cholangiocarcinoma using a non-invasive procedure is quite important. To find the possible tumor marker of CCA, we use the sera and label the compounds with fluorescent chemicals to try and find a certain compound found in the serum of patients with cholangiocarcinoma. One of our preliminary results showed that a new peak (named peak B) was found in 50% of CCA patients but in 6.3% in normal individuals[38]. In addition, Loilome *et al*[31] found that in liver fluke-associated CCA, PRKR1A overexpression is associated with an increased extracellular PKA autoantibody. The antibody titers in the sera from patients with CCA (0.154 ± 0.077), adenocarcinoma (0.150 ± 0.061) and OV infected individuals with fibrosis (0.157 ± 0.045) were significantly higher than that in healthy control subjects (0.129 ± 0.028), while there was no significant difference between the sera from OV infected individuals without fibrosis (0.139 ± 0.053) and that of healthy control subjects[31]. Recently, Matsuda *et al*[39] found the *Wisteria floribunda* agglutinin-positive mucin 1 and the L1 cell adhesion molecule[40] were sensitive biliary biomarkers for CCA. Silsirivanit *et al*[41] reported a novel Lewis a associated carbohydrate epitope, CA-S27, as a diagnostic and prognostic biomarker for CCA.

Although the prognosis of CCA is not good, there are several reports on the relationship of genetic changes and the prognosis of patients with CCA. One example would be with the classical comparative genomic hybridization studies. It was suggested that amplification of the D22S283 region of the chromosome was a favorable prognostic marker[42].

With recent rapid advancement of DNA sequencing technology, it becomes possible to analyze the whole genome sequence relatively less expensively. Therefore, it should be possible to search the responsible chromosomal region involved in the genetic determinants of the risk for CCA and the progression or inhibition of the growth of CCA in more detail. With the technology of genomics, proteomics and glycobiology, one can expect to find the high risk population for CCA more easily, to help the population better adjust their lifestyles for prevention of CCA and to detect the patients with CCA in its early phase.

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Management of acute severe ulcerative colitis

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Abstract

The management strategy of acute severe ulcerative colitis has evolved over the past decade from being entirely restricted to twin choices of intravenous steroids or colectomy to include colon rescue therapies like cyclosporin as well as infliximab. However it still remains a medical emergency requiring hospitalization and requires care from a multidisciplinary team comprising of a gastroenterologist and a colorectal surgeon. The frame shift in management has been the emphasis on time bound decision making with an attempt to curtail the mortality rate to below 1%. Intravenous corticosteroids are the mainstay of therapy. Response to steroids should be assessed at day 3 of admission and partial/non-responders should be considered for alternative medical therapy/surgery. Medical rescue therapies include intravenous cyclosporin and infliximab. Cyclosporin is administered in a dose of 2 mg/kg per day and infliximab is administered as a single dose intravenous infusion of 5 mg/kg. Approximately 75% patients have short term and 50% patients have long term response to cyclosporin. Long term response to cyclosporin is improved in patients who are thiopurine naïve and are started on thiopurines on day 7. Infliximab also has a response rate of approximately 70% in short term and 50% in long term. Both cyclosporin and infliximab are equally efficacious medical rescue therapies as demonstrated in a recent randomized control trial. Patients not responding to infliximab or cyclosporin should be considered for colectomy.

Key words: Ulcerative colitis; Acute severe colitis; Intravenous steroids; Cyclosporin; Infliximab

Core tip: The mortality of severe ulcerative colitis has drastically reduced from 30%-60% in pre steroid era to 1%-2.9% at present. However these figures are for specialist centers and at peripheral centers the mortality figures may be higher. The objective of this review is to provide in depth information for what can be categorized as a gastrointestinal medical emergency with the hope that informed clinical practices may translate to superior patient care at tertiary as well as peripheral centers treating ulcerative colitis. This review provides time bound framework, which looks at stepwise management of acute severe ulcerative colitis and explores the recent concepts of choice between biologics and cyclosporin colon rescue therapies in case of steroid refractory disease.

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INTRODUCTION

Acute severe ulcerative colitis (UC) is a medical emergency characterized by[1] (Table 1) presence of more than 6 bloody stools/d along with any one of the following: tachycardia > 90 bpm, fever > 37.8 ℃, Hb < 10.5 gm/dL, and/or ESR > 30 mm/h (Truelove and Witt's criteria). Other indices for defining severity include modified Mayo's classification[2], which is a combination of clinical and endoscopic findings, and Montreal classification[3], which is primarily based on Truelove and Witt's criteria.

Table 1 Modified Truelove and Witt's criteria for classification of severity of ulcerative colitis

	Mild	Moderate	Severe
Bloody stools per day	< 4	4-6	> 6
Pulse	< 90 bpm	≤ 90 bpm	> 90 bpm
Temperature	< 37.5 ℃	≤ 37.8 ℃	> 37.8 ℃
Hemoglobin	> 11.5 gm/dL	≥ 10.5 gm/dL	< 10.5 gm/dL
ESR	< 20 mm/h	≤ 30 mm/h	> 30 mm/h
CRP	Normal	≤ 30 mg/dL	> 30 mg/dL

However, Truelove and Witt's criteria is the most widely accepted disease severity index in clinical practice. The term acute severe colitis is preferred over fulminant colitis because the term fulminant is not well defined. It was coined in 1950 when it meant that single attack of UC could lead to mortality within 1 year[4], which is no longer relevant today. Approximately 20% UC patients with initial disease flares have severe UC[4], and about 15% patients have a severe attack at some stage of their disease[5]. Megacolon refers to presence of dilated colon (> 5.5 cm) on a plain abdominal X-ray film. Toxic megacolon is presence of megacolon with signs of systemic toxicity (fever, tachycardia, hypotension, leukocytosis). The overall lifetime incidence of toxic megacolon in patients with UC is 1%-2.5%[6]. Prior to introduction of corticosteroid therapy, mortality with acute severe UC was reported to be upto 22%-75% within first year of diagnosis[7]. First clinical trial of steroids for severe UC was performed in the 1950s and this trial reported a mortality of 7% in patients treated with steroids compared with 24% in the placebo group[8]. The mortality with severe UC has reduced to < 1% in specialist centers.

APPROACH TO MANAGEMENT

Investigations required at admission

In addition to monitoring patient's clinical feature and vital signs, all patients should have their full blood counts, liver and kidney function tests, electrolytes including serum magnesium and inflammatory markers [C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR)]. At least 3 stool samples for *Clostridium difficile* (*C. difficile*) toxin should be obtained to rule out superimposed pseudo-membranous colitis[9]. A plain abdominal X-ray should be done to exclude megacolon. Plain radiograph can also provide information about the extent of disease and can also predict response to treatment. The distal distribution of fecal residue can provide a rough estimate of disease extent as it correlates with the proximal extent of disease[10]. The predictors of poor response to treatment on a plain abdominal radiograph are presence of mucosal islands which are small, circular opacities that represent residual mucosa isolated by surrounding ulceration, or presence of more than two gas-filled loops of small bowel[11]. Flexible unprepared sigmoidoscopy with minimal air insufflation should be performed to confirm the diagnosis and exclude superimposed infection, especially cytomegalovirus (CMV) colitis[12]. Endoscopic markers of severe disease activity include hemorrhagic mucosa with deep ulceration, mucosal detachment on the edge of these ulcerations, and well like ulcerations[13].

Treatment

General management: In addition to specific therapy these supportive measures are very important in the management of patients with acute severe UC. These include: (1) Monitoring and replacement of intravenous fluid and electrolytes to correct and prevent dehydration or electrolyte imbalance as hypokalaemia/hypomagnesaemia can precipitate toxic dilatation[6]; (2) Anticholinergic, antidiarrheal, non-steroidal anti-inflammatory drugs and opioid drugs should be promptly withdrawn as these may precipitate colonic dilatation; (3) Malnourished patients should receive adequate nutritional support. Enteral nutrition is most appropriate and is preferred over parenteral nutrition as it is associated with significantly fewer complications than parenteral nutrition in acute colitis[14]. There is no evidence that bowel rest with parenteral nutrition alters the outcome[15]; (4) Flexible unprepared sigmoidoscopy and biopsy should be done to confirm the diagnosis of acute severe UC and exclude infections[16] such as CMV. Presence of active CMV infection is indicated by presence of cytomegalovirus inclusion bodies on colonic biopsies. However inclusion bodies are not very frequent even in patients with active disease with a sensitivity as low as 37.5%[17]. Special immunohistochemical staining against immediate early antigens of CMV increases the diagnostic sensitivity of histologic examination for CMV. In addition positive plasma real time PCR assays for CMV DNA at levels > 20 copies/100 μL is also an indicator of active CMV disease[18]. Presence of active CMV disease requires treatment with ganciclovir, especially if the patient is slow to respond to conventional therapy; (5) Stool analysis (in atleast 3 stool samples) to exclude co-existing *C. difficile* toxin is required especially in patients with history of prolonged hospitalization[19]. *C. difficile* infection co-existing with acute severe UC has been associated with increased morbidity and mortality, and requires appropriate antibiotic therapy (oral vancomycin or metronidazole)[20]; (6) There is increased risk of thromboembolic phenomena, in patients with active IBD compared to controls, especially during disease flares[21]. Therefore prophylaxis with subcutaneous low molecular weight heparin is indicated to reduce the risk of thromboembolism; (7) Topical corticosteroids or mesalazine may be administered if patient can tolerate and is able to retain them, although there have been no systematic studies in acute severe colitis; (8) Antibiotics are indicated only if infection is suspected or immediately prior to surgery. Controlled trials of antibiotics such as oral or intravenous metronidazole or ciprofloxacin in acute colitis have not shown any significant benefit in addition to conventional therapy[22,23]; and (9) Blood transfusion is indicated in patients with hemoglobin < 10 gm/dL[24].

In addition to these measures daily assessment

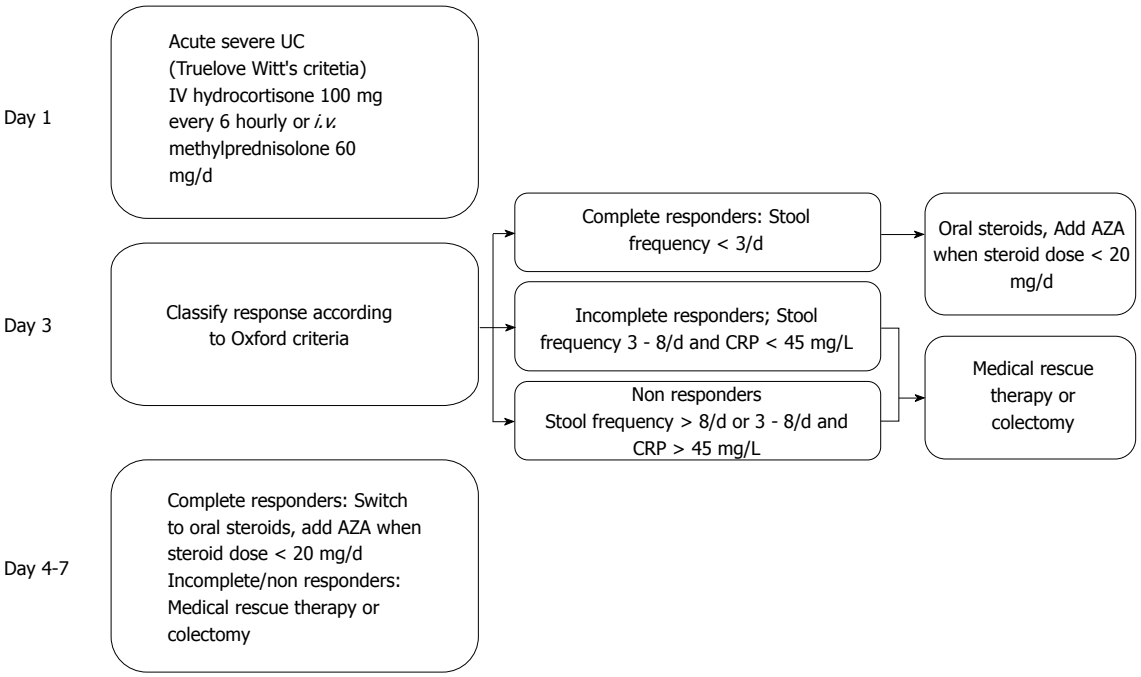

Figure 1 Algorithm for treatment decisions for patients with acute severe ulcerative colitis on intensive steroid therapy. AZA: Azathioprine.

of patients' clinical status should be done in following manner: (1) Physical examination is required daily to evaluate abdominal and rebound tenderness. Joint collaboration between medical and surgical team is required for appropriate management of such patients; (2) Vital signs should be recorded four times daily and more often if deterioration is noted; (3) A stool chart which records the number and character of bowel movements, including the presence or absence of blood and liquid versus solid stool should be properly maintained; (4) Measurement of blood count, CRP, serum electrolytes, serum albumin, liver function tests, and glucose should be done every 24 h; and (5) Abdominal radiographs should be done daily, especially in patients in whom there are signs of colonic distension and/or there is significant deterioration in clinical condition or laboratory parameters.

CORTICOSTEROIDS

Corticosteroids are the mainstay of therapy for acute severe UC. Steroids are given intravenously with methylprednisolone given in a dose of 60 mg/d or hydrocortisone 100 mg every 6 h. Treatment duration is usually limited to 7 to 10 d; continuing corticosteroid treatment beyond that period carries no additional benefit[25]. Truelove and Jewell published the first clinical trial of intravenous corticosteroids for acute severe UC in 1974[26]. Of 49 patients treated with intravenous steroids, 36 (73%) achieved complete remission by day 5. In a recently published systematic review of 1991 patients from 1974 to 2006[25], overall response to steroids was 67%. The overall short-term colectomy rate was 29% (565/1991) and mortality was 1%.

Predictors of response to steroids

Response to steroids is indicated by improvement in patients' symptoms (decreased stool frequency, urgency and rectal bleeding, improved stool consistency, reduction in abdominal pain, and improvement in general well being) and improved laboratory parameters (reduced CRP and ESR and improvement in hemoglobin and albumin).

At day 3 of admission, response to steroids should be measured by assessing stool frequency and CRP levels (Figure 1). In the landmark study by Travis *et al*[10], which included patients with 51 episodes of severe UC, presence of more than 8 stools/d or 3-8 stools/d plus a CRP > 45 mg/L at day 3 predicted a colectomy rate of 85%. In another prospective study by Lindgren *et al*[27] which included 97 episodes of severe UC, the following mathematical model was devised to predict colectomy: number of stools/d + 0.14 × CRP (mg/L) ≥ 8 predicted a colectomy rate of 72%.

Therefore regular assessment of response to steroids is of paramount importance in treating patients with acute severe UC. In a group of 80 patients who underwent emergency colectomy for severe UC between 1994 and 2000 in Oxford[28], patients with significantly longer duration of preoperative medical therapy (> 8 d) were more likely to have major post-operative complications.

Therefore at day 3 of admission, in cases of non response to steroids according to above mentioned criteria (stool frequency > 8/d or stool frequency 3-8/d and CRP > 45 mg/L) other treatment options or surgery should be considered. In cases of partial response, therapy should be continued till day 5-7, and if the patient still does not respond, other therapies/surgery should be considered (Table 2). In patients who respond to steroids, oral steroids should be started after 5-7 d of

Table 2 Ten year follow up of patients of Oxford cohort categorized at day 7 of intensive therapy

Parameter	Complete responders	Incomplete responders
Colectomy rate at 1 yr	5%	54%
Number requiring colectomy	6/19 (32%)	10/13 (76.9%)
Maximum steroid free remission	3.5 yr	< 1 yr

intensive therapy.

There are several other studies which have predicted response to steroids in acute severe UC. Ho *et al*[29], in a retrospective study found that, number of stools/day (score 1-4); hypoalbuminaemia < 3 mg/dL (score 1) and colonic dilatation > 5.5 cm (score 4) were useful in predicting colectomy as 85% of patients with a score ≥ 4 required colectomy. In another study by Ananthakrishnan *et al*[30], anemia, malnutrition, need for blood transfusion and total parenteral nutrition would independently predict colectomy. Radiological markers which can predict colectomy include the presence of mucosal islands on a plain abdominal radiograph which is associated with a 75% colectomy rate[31], and presence of an ileus (indicated by 3 or more small bowel loops of gas) which is associated with 73% colectomy rate[11]. In a study, presence of deep ulcers on endoscopy after gentle air insufflation identified 42/49 patients who required colectomy[32].

CYCLOSPORIN

Two controlled clinical trials established the efficacy of intravenous cyclosporin (fungal calcineurin inhibitor) as medical rescue therapy for acute severe UC not responding to intravenous corticosteroids. In the first landmark trial by Lichtiger *et al*[33] 9 out 11 patients in the cyclosporin (4 mg/kg per day) group had a response *vs* none of 9 placebo treated patients. The trial was terminated early for ethical reasons because of marked response to cyclosporin. Of nine placebo treated patients 5 patients were crossed over to cyclosporin and all five responded. In another study 73 patients were randomized to 4 mg/kg *vs* 2 mg/kg of intravenous cyclosporin[34]. Response rates at 8 d were similar in both groups (83% and 82% respectively), with 9% and 13% colectomy rate in 2 and 4 mg/kg group respectively. Therefore, cyclosporin dose of 2 mg/kg per day has become the standard in clinical practice. Another European study compared intravenous cyclosporin (4 mg/kg) with intravenous steroids and found similar response rates between the two groups (64% *vs* 53%)[35]. Therefore cyclosporin monotherapy may be preferred over steroids in patients who have high chances of side-effects with steroids including patients with osteoporosis, poorly controlled diabetes and those who are susceptible to steroid-psychosis. Overall, pooled results from controlled and non-controlled trials show response rates with intravenous cyclosporin to vary from 76% to 85%, with median time to response being 4 d[35].

Table 3 Long term response rates to cyclosporin[38]

Initial response	74%
1 yr	65% relapsed
3 yr	90% relapsed
7 yr	58% colectomy rates

However, one of the major limitations associated with cyclosporin use is its side effect profile. The short-term side effects are a cause of concern because cyclosporin is generally used as bridge to immunomodulators. These include minor side effects, which occur in 31%-51% patients, including tremors, paresthesias, malaise, headache, abnormal liver function tests, gingival hyperplasia and hirsutism. Major complications are reported in 0%-17%; including hypertension, renal impairment, infections and neurotoxicity[36]. Cyclosporin therapy in UC is associated with a mortality rate of approximately 1.8%-3.5%[36]. Therefore, following points should be considered before starting cyclosporin therapy.

Cyclosporin should not be used if cholesterol < 115 mg/dL or magnesium < 1.4 mEq/L. It should also be avoided in presence of hypertension, renal impairment, epilepsy, sepsis, age > 80 years. Magnesium, cholesterol, and creatinine should be measured at baseline and within 48 h of starting cyclosporin.

Cyclosporin should be administered in a dose of 2 mg/kg per day intravenously aiming for levels 150-250 ng/mL[37].

Oral microemulsion 4 mg/kg twice daily can be alternatively considered.

Blood Pressure and renal function should be monitored and cyclosporin should be stopped if serum creatinine rises > 25%.

Cyclosporin should be stopped if there is no improvement in 7 d.

In responders intravenous cyclosporin (Figure 2) should be switched to oral cyclosporin 4 mg/kg per day twice daily. Monitoring of trough levels (150-250 ng/mL) should be regularly done. Azathioprine should be started along with oral cyclosporin. Cyclosporin should be stopped after 3 mo.

Infective complications with cyclosporin can be avoided by minimizing concomitant immunosuppressants and by using prophylactic antibiotics when indicated.

Regarding long term efficacy of cyclosporin (Table 3) several cohorts have been evaluated long term colectomy in patients treated with cyclosporin. In the retrospective cohort from Oxford, 42% patients could avoid colectomy after 7 years[38]. Overall, approximately 50% patients will avoid colectomy over a period of 2-3 years[39,40]. Immunomodulators when used with cyclosporin can decrease the colectomy rate, thus improving the long term efficacy of cyclosporin. In a study by Cohen *et al*[41] probability of avoiding colectomy at long-term follow-up (5.5 years) was 66% in patients receiving cyclosporin and azathioprine/mercaptopurine compared with 40% in those who received cyclosporin alone. Further studies in this regard

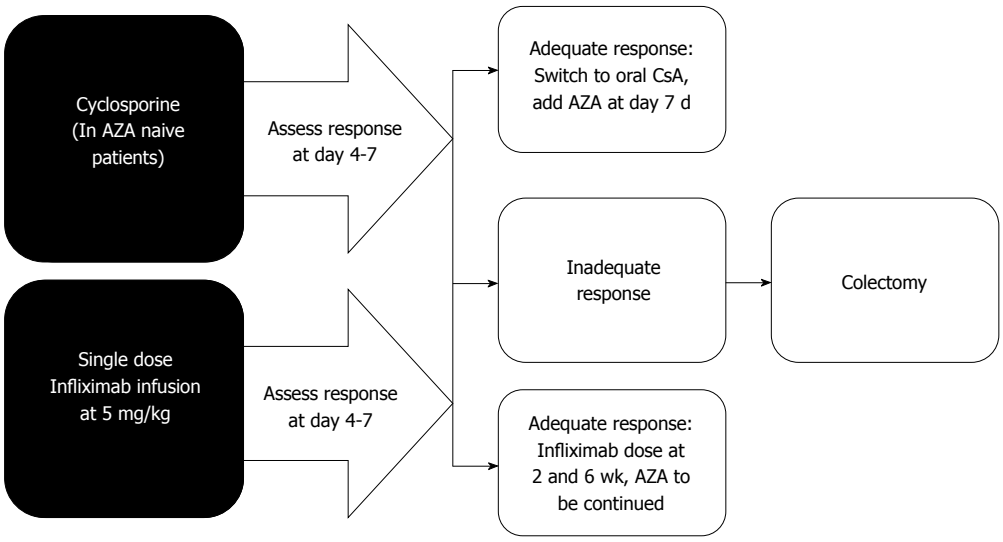

Figure 2 Algorithm for medical rescue therapy after failure of response to intravenous steroids.

have shown that in patients already on immunomodulators at the time of admission with acute severe UC, the likelihood of needing a colectomy following treatment with cyclosporin is higher than among those in whom immunomodulators are started after admission[40].

Therefore, cyclosporin is more beneficial in patients with acute severe UC who are thiopurine naïve at the time of admission. In patients who are already on thiopurine at the time of admission, the outcome with cyclosporin would be less favourable and other medical options or surgery needs to be considered.

INFLIXIMAB

Infliximab the chimeric monoclonal antibody against tumor necrosis factor (TNF) alpha has been found to have a favorable response in patients with steroid refractory acute severe UC. In an open label study of 6 steroid refractory severe UC patients[42], single infusion of infliximab in a dose of 5 mg/kg showed marked clinical improvement at day 7 in all patients. Four out of these 6 patients were in long term remission at median follow up of 5.5 mo. Later a randomized placebo controlled trial of 45 patients (24 infliximab and 21 placebo) showed that a colectomy rate at 3 mo was significantly lower in infliximab group as compared to placebo group (29% *vs* 67%, $P = 0.017$)[43]. The maximum benefit of infliximab was seen in patients with moderately severe disease than in those with most severe disease. Prior exposure to thiopurines does not seem to affect the outcome of patients treated with infliximab[43]. Other factors which may adversely affect outcome with infliximab include increased baseline CRP (> 20 mg/L), concomitant steroid use, disease duration ⩽ 3 years and baseline Mayo score ⩾ 10[44]. Screening for infections and immunization history should be obtained prior to initiating infliximab therapy. Screening tests which need to be done include hepatitis B serology, HIV serology, chest radiograph and tuberculin skin test or Interferon gamma release assays for latent tuberculosis.

Long term follow up data up to 3 years in infliximab treated severe UC patients are available. Two studies with follow up data of 1 year show colectomy rates of approximately 25% at 1 year in infliximab treated patients[45,46]. In another Swedish study, colectomy rate at 3 years in infliximab treated patients was 50% as compared to placebo (76%)[47].

There are no exclusive trials of other anti TNF agents for acute severe UC. However, there are few trials of adalimumab in moderate to severe active UC which showed efficacy of adalimumab over placebo. Reinisch *et al*[48] showed that adalimumab induced remission in 18.5% patients as compared to 9.2% patients in placebo group ($P = 0.031$). In another study Sandborn *et al*[49], in a similar group of patients showed efficacy of adalimumab over placebo (16.5% *vs* 9.3%, $P = 0.019$) in inducing remission.

CYCLOSPORIN *VS* INFLIXIMAB

Before the landmark randomized trial CYSIF (Cyclosporin With Infliximab in Steroid-refractory Severe Attacks of Ulcerative Colitis) between cyclosporin and infliximab there was limited evidence to suggest any difference in efficacy of cyclosporin and infliximab. In a retrospective review of two cohorts (43 treated with cyclosporin and 49 treated with infliximab) there was lower short term colectomy rate in the cyclosporin group[50]. The CYSIF trial[51] randomized 111 thiopurine naïve patients with severe UC after 5 d of IV steroids to cyclosporin (2 mg/kg per day for 8 d followed by 4 mg/kg per day orally) and infliximab (5 mg/kg *iv* infusion at 0, 2 and 6 wk). Patients who responded at day 7 received oral azathioprine and tapered steroids from day 8. The response to treatment at day 7 was seen in approximately 85% patients in both groups. Colectomy rates at day 98 were also similar be-

Table 4 Mortality according to day of surgery after intensive steroid therapy

Timing of surgery Total emergency surgeries = 72	Number of patients	Mortality
Overall	51	8
≤ 5 d	17	0/17
> 5 d	34	8/34

tween cyclosporin and infliximab (18% *vs* 21%, $P = 0.66$). Treatment failure at day 98 was also similar, seen in 60% patients in the cyclosporin group *vs* 54% in the infliximab group. There was no clear evidence of superiority of any one therapy over other.

Therefore choosing between cyclosporin and infliximab depends upon physician and patient preferences as both appear to be equally efficacious in the setting of acute severe colitis.

SWITCHING BETWEEN INFLIXIMAB AND CYCLOSPORIN

In cases of non-response to infliximab or cyclosporin, switching to either therapy is associated with significant morbidity and mortality and is not recommended. In the largest study of 86 patients on this aspect, 65 patients were administered infliximab after cyclosporin and 21 patients had cyclosporin after infliximab. Thirty three percent patients underwent colectomy within 3 mo and 1/3rd of the patients had adverse effects in form of infections[52].

TACROLIMUS

Tacrolimus is also a calcineurin inhibitor with mechanism of action similar to that of cyclosporin. A randomized trial of tacrolimus included 27/60 patients with severe UC[53]. In this trial partial response was seen in 67% patients, although complete remission was not seen on any patient. However, further case series have shown results similar to that of cyclosporin[54,55].

TOXIC MEGACOLON AND OTHER COMPLICATIONS OF SEVERE UC

Toxic megacolon may be defined as colonic dilatation of more than 5.5 cm along with signs of systemic toxicity. Lifetime incidence of toxic megacolon in patients with UC varies from 1%-2.5% and approximately 5% severe UC patients who are hospitalized may develop toxic megacolon[6]. Risk factors include dyselectrolytemia, full bowel preparation and medications (antidiarrheal, anticholinergic, and opiods)[6]. Earlier identification of this condition, prompt institution of medical therapy (nil per oral, intravenous broad spectrum antibiotics, fluid and electrolyte management, and intensive therapy) and low threshold of surgery in cases of non-response to medical therapy within 48 h will decrease the morbidity and mortality of this condition.

Other complications include perforation which is the most serious complication of severe UC. Risk factors include inappropriate total colonoscopy and delaying treatment of toxic megacolon. Diagnosis of perforation can often be delayed as abdominal signs can be masked when patient is on steroids. Therefore, patients with severe UC should be monitored closely for abdominal signs and on the slightest suspicion abdominal radiographs should be obtained. Other complication includes severe hemorrhage.

SURGERY

Surgery is the final option for patients with severe UC not responding to medical therapy. Other indications for surgery include toxic megacolon, perforation and severe haemorrhage. The decision for surgery should not be delayed as this increases the morbidity and mortality of surgery. In a study performed at our center, the mortality of emergency surgery was very high if the intervention was delayed beyond 5 d following non-response to intravenous steroid therapy (Table 4)[56]. In another study from Oxford, higher surgical complication was noted if surgery was delayed beyond 8 d of medical therapy[28]. Therefore management of severe UC requires close collaboration between surgeon and gastroenterologist so that appropriate decisions can be taken without delay.

Most centers advocate a 3 step surgery in emergency setting. The surgical procedure of choice in acute setting is sub-total colectomy and ileostomy, with the rectum left in situ. The whole of rectum and inferior mesenteric artery should be preserved, which facilitates further surgery. The bowel can either be closed in subcutaneous fat or brought forward as mucous fistula, depending upon the surgeon's decision. Subtotal colectomy is a safe procedure even in critically ill patients[57,58] and will relieve the patient from burden of severe colitis, thus allowing the patient to normalise health and nutrition. Reconstructive surgery is best performed approximately 6 mo after primary surgery[59]. The second step consists of ileal pouch formation and defuctioning temporary ileostomy. In the final step ileal pouch anal anastomosis (IPAA) is done restoring normal continuity.

There appears to be a strong association of prolonged use of immunosuppression and poor wound healing after surgery which may manifest as wound dehiscence, infection following intestinal leak or a pelvic abscess following anastomotic leak. Long-term preoperative steroid use has been found to be a significant risk factor for anastomotic leak. Immunosuppressive agents (azathioprine and 6-mercaptopurine) have not been associated with increased postoperative complications. When used alone, cyclosporin has not been associated with increased postoperative complications. The use of infliximab (IFX) and its impact on postoperative course is debatable and is a subject of intense interest. Two studies have identified a relationship between IFX and postoperative complica-

tions in IPAA patients. The first report came from Mayo Clinic[60] which included a retrospective survey of 47 patients who received preoperative IFX and 254 who did not. In the multivariate analysis, IFX was independently associated with increased risk of pouch-related and infectious complications. The authors concluded that IFX was a surrogate for critical patients who were at a higher risk for postoperative complications. The second study by Mor *et al*[61], had a case control design. It suggested that patients who had preoperative IFX were 3.5 times more likely to experience an early postoperative complication as compared to control patients. IFX-exposed patients were nearly 14 times more likely to suffer infectious complications. Other studies, which have not been in agreement with the conclusion of above-mentioned studies, include a large retrospective review of 413 patients with UC and CD over a 14-year period[62]. This study did not find any association between IFX and postoperative complications. The study faced certain criticisms, which included a heterogeneous population with > 50% of patients having CD and only 26 patients with UC who had a preoperative exposure to IFX. Another study[63], evaluating surgical outcomes in 141 UC patients over a 10-year period, found no association of IFX exposure with postoperative complications. In the same study, steroid use was related to increased infectious complications. The limitation of this study was that only 22 patients had IFX exposure prior to surgery. A recent meta-analysis concluded that infliximab use is not associated with increased risk of post-operative complications[64]. At present, no firm conclusions can be drawn. All these studies suffer from a retrospective design. Moreover, it is possible that patients who require IFX represent a patient population, which is at a higher risk for postoperative complications. At the same time, evidence exists that IFX may have a causal role in impairing wound healing and causing anastomotic failure and pelvis sepsis. The definite conclusion which can be drawn is that in patients who have received IFX, a three-stage procedure for IPAA should be considered rather than a two-stage procedure.

Mortality rates associated with emergency colectomy are higher as compared to elective colectomy[65]. In a study from England which included more than 20000 patients with IBD, mortality rates for patients with UC 3 years after colectomy was significantly lower with elective as compared to emergency colectomy (3.7% *vs* 13.2%)[66]. Surgery is not the preferred modality of therapy in young females as ileal pouch anal anastomosis has been associated with lower fertility and fecundity rates[67,68]. In patients with severe malnutrition, surgery may have to be deferred as the risk of post operative complications is significantly increased in this setting.

CONCLUSION

Acute severe ulcerative colitis as defined by Truelove Witt's criteria is a medical emergency that requires immediate hospitalization. Fluid and electrolyte balance, withdrawl of drugs promoting colonic dilatation and adequate nutritional support are important adjuncts in the management of severe UC. Intravenous corticosteroids are the first line therapy for severe UC, and approximately two thirds of patients respond. Response to steroids should be assessed at day 3, and in non-responders/partial responders, medical rescue therapy or surgery should be considered. Efficacy of both cyclosporin and infliximab in this setting is comparable as shown in a recent randomized trial. A close coordination between gastroenterologist and surgeon is required for optimal management of severe UC. Surgery is always an option after failure of IV steroids, and all patients should be given an option of surgery. A time bound strategy is required to manage such patients and surgery should not be delayed beyond 5 d of intensive therapy, as a delay increases surgical morbidity and mortality.

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LETTERS TO THE EDITOR

Antiviral treatment in patients with *cytomegalovirus* positive ulcerative colitis

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Abstract

Cytomegalovirus (CMV) is a common virus in patients with ulcerative colitis receiving immunosuppressive drugs. Many studies suggested that CMV infection is an exacerbating factor in patients with ulcerative colitis. The role of CMV in exacerbations of ulcerative colitis has been discussed. One of studies starting this discussion is an article entitled "CMV positive ulcerative colitis: A single center experience and literature review" by Kopylov *et al*. However, we think that there are some points that should be emphasized about the study. Especially, the small number of patients in the study has led to meaningless results. Large controlled prospective trials are needed to clarify the benefit of antiviral therapy for active ulcerative colitis patients.

Key words: *Cytomegalovirus*; Ulcerative colitis; Antiviral treatment; Steroid resistant; Colonoscopy

Core tip: Many studies suggested that *cytomegalovirus* (CMV) infection is an exacerbating factor in patients with ulcerative colitis. The role of CMV in exacerbations of ulcerative colitis has been discussed. We believe that large controlled prospective trials are needed to clarify the benefit of antiviral therapy for active ulcerative colitis patients.

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TO THE EDITOR

We read with great interest the recently published article entitled "Cytomegalovirus (CMV) positive ulcerative colitis: A single center experience and literature review" by Kopylov *et al*[1] in the February 15, 2013 issue of *World Journal of Gastrointestinal Pathophysiology*. In this retrospective study, the authors compared the clinical outcomes of CMV-positive ulcerative colitis patients with and without antiviral therapy (gancyclovir). They concluded that patients with obvious histological evidence of CMV in the colonic mucosa may not universally require antiviral therapy and may respond to conventional anti-inflammatory therapy. This study reveals the indications for antiviral therapy in CMV-positive patients with ulcerative colitis. Moreover, it provides some new information that represents educational "take-home messages" for readers. We believe that further studies will be performed in light of these findings. However, we think that there are some points that should be emphasized about the study.

First, in the discussion section of the paper, the authors reported that patients in the antiviral-treated group "are in greater need of hospitalization" than patients without antiviral treatment. However, as shown in Table 1, no statistically significant difference could be seen between these two groups. As we know that the *P* value is revealed below a certain significance level, often 0.05, this elucidates a strong presumption against the null hypothesis[2,3]. In light of this, we suggest that the conclusion of

Table 1 Clinical and demographic characteristics of the included patients (mean ± SD)

Patient characteristic	Treated (n = 7)	Untreated (n = 6)	P value
Age (yr)	50.0 ± 14.6	45.0 ± 13.6	0.540
Gender (male/female)	4/3	3/3	0.400
Extent of disease			
Pancolitis	6	5	0.540
Left-sided	1	1	0.540
Age at diagnosis of UC, yr	35.7 ± 13.3	41.5 ± 13.3	0.530
Duration of disease, yr	14.2 ± 9.3	3.5 ± 1.8	0.008
Hospitalized patients	6	4	0.560
Prehospitalization treatment			
SC	4	2	0.560
Thiopurines	3	2	1.000
Infliximab	1[1]	0	1.000
5-asa	5	4	1.000
SC + thiopurines	2	1	1.000
Treatment during hospitalization			
SC	6	3	0.400
Infliximab	1	0	1.000
Cyclosporine	3	0	0.200
Timing of colonoscopy (d)	3.8 ± 2.4	2.7 ± 3.4	0.600
Positive cytopathic changes on HE staining	2	0	0.460
Hospitalization outcome			
Death	1	0	1.000
Colectomy	1	0	1.000
Outcome by the end of the follow-up			
Colectomy	3	0	0.190
Death	1	0	1.000

[1]Combined with systemic corticosteroids and thiopurine. Treated: Patients who received antiviral therapy; Untreated: Patients who did not receive antiviral therapy; Timing of colonoscopy: Number of days from hospital admission; SC: Systemic corticosteroids; HE: Hematoxylin and eosin; IHC: Immunohistochemistry; UC: Ulcerative colitis.

the present study should be reviewed.

Second, the authors mentioned in the discussion section that only three patients without antiviral therapy were hospitalized. However, four patients in the group without antiviral therapy were hospitalized, according to Table 1. Finally, there are conflicting data regarding the staining method of the histopathological examination. Consequently, we conclude that, before making certain interpretations, this work should be rearranged in light of the above-mentioned suggestions. This could provide the readers of the journal clearer information regarding the role of CMV infection in the pathogenesis and clinical course of ulcerative colitis.

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